Mechanisms of Glucocorticoid Programmed Disease

by

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Declaration

I declare that this thesis was written by me and that the data presented within it are a result of my own work, except where outlined specifically in the text.

No part of this work has been submitted for any other degree.

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Acknowledgements

Men go forth and wonder at the heights of mountains, the huge waves of the sea, the broad flow of rivers, the vast compass of the ocean, the courses of the stars; and they pass by themselves without wondering.

St. Augustine, *Confessions*,
Book X, chapter 8.

I am exceedingly grateful to the Cardiovascular Research Initiative for affording me the opportunity to wonder and question, and to the Wellcome Trust for generously funding these efforts. Immense thanks to all the scientists and clinicians who shared their astonishing knowledge and experience with me, and to all of those who cleared my head of nonsense and filled it with better ideas. I should like to especially acknowledge all the staff of the Endocrinology Unit and the Biological Research Facility for their constant and unfettered generosity. In particular, my supervisors' advice and support — scientific, literary and career — has been invaluable throughout; and I shall continue to express and propagate their invaluable *memes* in my daily life. Above all and for everything, I thank my family, especially Mom, Fergus and Betty; my extraordinary friends, most notably my 'family' in Edinburgh, Eimear and Meera — whether one at a time or all together you continue to reveal wonders I never knew.

Abstract

Substantial epidemiological evidence correlates low weight or thinness at birth with increased risk of disease in later life; notably insulin resistance, hypertension and ischaemic heart disease. This concept of intrauterine life events having permanent influences upon later health has been termed 'programming'. Whilst the molecular mechanisms linking these effects are unknown, overexposure of the foetus to glucocorticoids has been implicated. Treating pregnant rats with dexamethasone (DEX), a synthetic glucocorticoid commonly used in obstetric practice, results in offspring born of low weight, who subsequently develop adulthood hypertension, glucose intolerance and insulin resistance. Whilst prenatal DEX-programmed glucose intolerance is associated with permanently increased hepatic activity of a key gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK), mechanisms underlying the programming of hypertension remain unidentified. DEX-programmed hypertension occurs in both sexes, whilst hyperglycaemia/hyperinsulinaemia has only been demonstrated in male offspring. Principally, this thesis investigates the role of the renin-angiotensin (RAS), and sympathetic nervous systems (SNS) in determining programmed hypertension, and seeks to determine whether programming effects are sexually dimorphic. It further examines the impact of dietary manipulations, and environmental noise stress, on prenatally-treated offspring phenotypes.

DEX administration in the last week of gestation reduces offspring birth weight and programmes adult cardiovascular and metabolic physiology in a sex specific manner. In male offspring, prenatal glucocorticoid exposure programmes elevated basal circulating corticosterone, elevated PEPCK activity, and produces adulthood post-glucose hyperglycaemia and hyperinsulinaemia. Whilst in female offspring, prenatal DEX programmes elevated hepatic angiotensinogen mRNA expression, elevated plasma angiotensinogen and renin activity, and produces hypertension, when measured by tail-cuff plethysmography.

A 4-fold reduction in dietary sodium intensifies this RAS dysregulation in female DEX-treated offspring; however this does not exacerbate their programmed blood pressure phenotype. Conversely, the lower sodium diet results in hypertension

in prenatally vehicle treated animals, and supports a role for both the HPA and RAS in mediating this. Furthermore, acute exposure to the lower sodium diet is sufficient to cause glucose intolerance and insulin resistance in female adult rats, irrespective of their prenatal treatment.

Unlike previous studies, offspring blood pressure was subsequently assessed with radiotelemetry, which is unmarred by any stress artefact. We now show that prenatal DEX-treated male and female offspring actually display ***lower*** basal blood pressure in adulthood; with the commonly expected hypertensive phenotype only being noted when these offspring are subjected to any stressor, regardless of its apparent banality. Moreover, DEX-treated offspring sustain this stress-induced hypertension for longer. These hypertensive responses are mediated by alterations in the responsivity of the sympathetic nervous system, being ameliorated by the inhibition of catecholamine synthesis, and further exaggerated by the promotion of systemic catecholamine release. Additionally, we demonstrate that DEX-treated offspring display greater sensitivity to various vasoconstrictors in the isolated mesenteric vasculature.

Finally, perinatal exposure of pregnant rats to environmental noise pollution results in vehicle-treated offspring with a phenotype analogous to prenatal DEX-treated offspring i.e. lower birth weight, hypercorticosteronaemia, hypertension, and features of the insulin resistance syndrome. Conversely, exposure of DEX-treated offspring to the same perinatal noise stressors does not appear to further influence their phenotype. Therefore, perinatal stress produces a similar phenotype to prenatal glucocorticoid over-exposure.

These findings demonstrate that *in utero* over-exposure to glucocorticoids actually results in stress-induced hypertension, and support a role for both RAS and SNS in mediating this. Furthermore, it appears that the programming of cardiovascular physiology may reflect distinct processes in each gender, whilst the programming of metabolic physiology is male specific.

CONTENTS

List of Abbreviations

A

Ab	Antibody
AC	Adenylate cyclase
ACh	Acetylcholine
ACTH	Adrenocorticotrophic hormone
AngI	Angiotensin I
AngII	Angiotensin II
Aogen	Angiotensinogen
ANOVA	Analysis of variance
ANP	Atrial natriuretic peptide
ATP	Adenosine triphosphate
A.U.	Arbitrary unit(s)
AVP	Arginine vasopressin

B

bp	Base Pair
BP	Blood Pressure
BSA	Bovine Serum Albumin

C

$CaCl_2$	Calcium chloride
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribose nucleic acid
cGMP	Cyclic guanidine monophosphate
CBG	Corticosterone binding globulin
CC	Carotid cannulation
cort	Corticosterone
CNS	Central nervous system
cpm	Counts per million
CRH	Corticotrophin-releasing hormone

D

DAG	Diacylglycerol
Dex	Dexamethasone
dGDP	Deoxyguanosine diphosphate
dH_2O	Deionised water

DNA	Deoxyribo-nucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothriotol
dTTP	Deoxythymosine triphosphate

E

EDTA	Ethylene diamine tetra-acetate
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum

G

g	Centrifugal force
G	Gauge
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GDP	Guanidine diphosphate
GH	Growth hormone
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GTP	Guanidine triphosphate

H

11β-HSD	11β-Hydroxysteroid dehydrogenase
HPA-axis	Hypothalamic-pituitary –adrenal axis
hr(s)	Hour(s)

I

I.D.	Internal diameter
i.u.	International units
IMRA	Immunoradiometric assay
i.p.	Intra-peritoneal
IP_3	Inositol 1, 4, 5-trisphosphate
IUGR	Intrauterine growth retardation

K

KCl	Potassium chloride
KH_2PO_4	Di-Hydrogen potassium phosphate
KRB	Krebs'-Ringer bicarbonate

L

LB	Luria-Bertoni/ liquid broth
LSD	Least significant difference

M

$MgSO_4$	Magnesium Sulphate
min(s)	Minute(s)
MOPS	(3-[N-Morpholino]) propanesulphonic acid
MR	Mineralocorticoid receptor
mRNA	Messenger RNA

N

NA	Noradrenaline
NaCl	Sodium chloride
$NaHCO_3$	Sodium hydrogencarbonate
$NaSO_4$	Sodium sulphate
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide (reduced form)
NIDDM	Non-insulin dependent diabetes mellitus

O

O.D.	Outside diameter

P

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
PEPCK	Phosphoenolpyruvate carboxykinase
PIP_2	Phosphatidylinositol 4, 5-bisphosphate
PLC	Phospholipase C
PKA	cAMP-activated protein kinase
PKC	Phospholipase C-activated protein kinase
PRA	Plasma renin activity
PSS	Physiological saline solution

R

RIA	Radio-immunoassay

RNA	Ribo-nucleic acid
rpm	Revolutions per minute
RT	Reverse transcription
RTP	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction

S

SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFO	Subfornical organ
sGC	Soluble guanylate cyclase

T

t	Time
TCP	Tail-cuff plethysmography
TE	Tris-EDTA
TMB	Tetramethylbenzidine
Tris	Tris[hydroxymethyl]-aminomethane
tRNA	Transfer RNA

U

U	Unit
UTP	Uracil triphosphate
UV	Ultraviolet (light)

W

w/v	Weight per volume

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Introduction

1.1 Background

During the 9 months of gestational life, a fertilised egg, and later a foetus must negotiate a plethora of biological milestones, which are sculpted by its intrauterine environment. For example, without enough folate, a foetus can't build and seal a spine; a deluge of alcohol can interfere with the delicate wiring of its nervous system; and certain medications taken by the mother can be detrimental to foetal limb development. However, some effects of gestational sculpting may lie dormant for decades before rearing their heads.

Such was the conclusion drawn by Professor David Barker, who collected detailed birth records made by midwives early in the 20th century, and followed up the individuals concerned until middle age. Unexpectedly, adults who had been born with birth weights at the lower end of the normal range were found to have a higher risk of a constellation of adult disease, including hypertension and type II diabetes (Barker *et al.* 1989; Barker 1990). It was a startling idea. For years, it was commonly accepted that adulthood cardiovascular disease, such as hypertension, was due to the interplay between an adverse lifestyle and the individual's genetic background. Barker's pioneering epidemiological studies now suggested that the foetal environment is potentially as important as the adult one. His resulting 'Foetal origins of disease' hypothesis stated that "Foetal undernutrition in middle to late gestation, which leads to disproportionate foetal growth, programmes later....disease" (Barker 1995).

A flurry of research subsequently focused on the likely physiological and molecular mechanisms underlying these early life 'programmed' events. In our laboratory, we utilise a rodent model of foetal over-exposure to glucocorticoids to explain this phenomenon (Edwards *et al.* 1993; Seckl *et al.* 1999b). And indeed, the phenotype of our model recapitulates many of the features revealed by the human epidemiological studies, including low birth weight; adulthood hypertension, glucose intolerance, insulin resistance and altered behavioural responses (reviewed in Seckl 2001; Welberg & Seckl 2001; O'Regan *et al.* 2001). The aim of this thesis was to investigate the molecular mechanisms underlying the hypertension associated with prenatal glucocorticoid treatment, and to further determine whether programmed cardiovascular and metabolic events are sexually dimorphic.

1.2 Foetal Origins of Adult Disease

1.2.1 Governors of *in utero* development

In utero growth and development is principally governed by the genetic composition of the foetus, maternal physiology, and the placenta, which is not only responsible for selective change of materials between the two, but also serves as an endocrine organ. Placental function is paramount in delivering adequate oxygen and metabolic substrates to the foetus, whilst simultaneously removing any waste products. Growth of the placenta and foetus are not always synchronous, thereby varying demands on the placenta throughout gestation (Robinson *et al.* 1995). Whilst placental derived luteinising hormone, progesterone, and oestrogen are important in maintaining pregnancy, the placenta also secretes other hormones, amongst them corticotrophin releasing hormone (CRH) (Goland *et al.* 1993).

The foetal endocrine system is also crucial in governing intrauterine development. Whereas pituitary growth hormone assumes greater importance after birth, glucocorticoids, insulin and thyroid hormones all have vital prenatal effects. Insulin, which has little effect on tissue differentiation, stimulates foetal growth by increasing the mitotic drive and nutrient availability for tissue accretion (Fowden 1992). In contrast, thyroid hormones affect differentiation and proliferation of different tissues at different times during gestation, thereby having an asymmetric effect on growth. Glucocorticoids are mainly concerned with tissue differentiation and maturation aimed at preparing the foetus for postnatal life (Liggins 1994). They promote surfactant production, allowing postnatal lung inflation (Liley *et al.* 1988); mature the gastrointestinal tract (Trahair & Sangild 1997); and induce PEPCK transcription, essential to perinatal gluconeogenesis (Liggins 1976). Their levels rise towards the end of pregnancy, and are further involved in the initiation of parturition (Whittle *et al.* 2001). Endocrine effects most probably operate though the IGF system. Both IGF I (dominant after birth) and IGF II (dominant pre-birth) stimulate growth (Sara & Hall 1990), and glucocorticoids are essential to induce the switch between the two isoforms (Fowden 1995). Foetal hormones, therefore, promote growth and development *in utero* by altering both the metabolism and gene expression of foetal tissues. These endocrine actions ensure that foetal growth is proportionate to nutrient supply, and that prepartum maturation occurs in preparation for extrauterine life.

1.2.2 Early life origins of adult disease

Myriad epidemiological studies suggest that factors operating in early life are important determinants of the risk of common cardiovascular and metabolic disorders of adult life. Studies in distinct populations initially in the UK, but later also elsewhere in Europe, Asia, Australia, Africa, the Caribbean and the USA, have shown that low weight or thinness at birth increase the subsequent prevalence of hypertension, dyslipidaemia, insulin resistance/ Type 2 diabetes and ischaemic heart disease deaths in adult life (Barker 1990; Barker *et al.* 1993; Curhan *et al.* 1996a; Curhan *et al.* 1996b; Fall *et al.* 1995; Lithell *et al.* 1996; Leon *et al.* 1996; Forsen *et al.* 1997; Rich-Edwards *et al.* 1997). These 'early life' associations appear to be important predictors of adult disease increasing by 40% to 300% the risk of adult degenerative disease (Curhan *et al.* 1996a; Curhan *et al.* 1996b; Barker 1990).

1.2.3 Proposed hypotheses for and against the early life origins of adult disease

During critical periods of development, when cells are rapidly dividing, tissues become more susceptible to the effects of external stimuli, thereby creating 'windows' of opportunity for the environment to affect development (Widdowson & McCance 1975). These stimuli can permanently affect a wide range of physiological processes, ultimately resulting in later dysfunction and disease (Edwards *et al.* 1993; Barker 1999; Benediktsson *et al.* 1993). This phenomenon, referred to as 'programming', was first demonstrated in the late 1960s; when a single dose of androgen was discovered to permanently modify physiology, through altered development, but only if it was administered during one of these 'windows' (Arai & Gorski 1968).

Whilst the accumulation of epidemiological evidence linking low birth weight with adult disease continues to increase, we remain relatively ignorant of the underlying programming mechanisms. Nonetheless, certain alterations in maternal physiology have recently been demonstrated to be linked to, or cause, intrauterine growth retardation (IUGR) and subsequent deranged physiology and disease. Amongst the first and perhaps most renowned of these implicated maternal malnutrition (Godfrey *et al.* 1996; Roseboom *et al.* 2001b), and is discussed later (see section 1.3.3). However, a plethora of other programming factors have since been demonstrated; some acting prenatally e.g. cytokines (Dahlgren *et al.* 2001), and

others operating within the early postnatal period e.g. endotoxin treatment (Shanks *et al.* 2000) and physical handling of pups (O'Donnell *et al.* 1994).

If we recall the importance of endocrine control on foetal growth and development, we might expect perturbations in intrauterine hormone levels to act as programmers of disease. And, indeed, altered androgen (Gustafsson & Stenberg 1974a), oestrogen (vom Saal *et al.* 1997), and thyroid (Castello *et al.* 1994) levels all have permanent effects on reproduction, brain or metabolic function. However, perhaps the most intriguing and wide-ranging influences of hormonal programming are observed with prenatal overexposure to glucocorticoids (Benediktsson *et al.* 1993; Seckl 2001; O'Regan *et al.* 2001), which is discussed further below.

The 'Foetal Insulin Hypothesis' (Hattersley & Tooke 1999), provides an alternative explanation to the above. It suggests that specific genetic polymorphisms result in increased insulin resistance and impaired growth, and that it is these polymorphisms that underlie the association of birth weight and adult cardiovascular disease. Of course, this mechanism could operate in tandem with the previously discussed environmental effects. The role of genetics in linking birth weight to later disease is discussed in section 1.3.1.

Alternatively, it has been proposed that those who suffered intrauterine adversity and failed to grow, continue to be exposed to an adverse environment in childhood and later life and it is this later unfavourable environment that produces the effects attributed to programming *in utero* (Wannamethee *et al.* 1996). However, there is scanty evidence to support this contention. Rather, associations between low birth weight and elevated blood pressure or insulin resistance, are found in each social group, and are independent of adult 'lifestyle' influences such as smoking, alcohol intake and obesity (Barker 1995). These 'lifestyle' risk factors are additive to the influences of early life, such that, for example, the highest prevalence of Type 2 diabetes mellitus is seen in those people whom were small at birth but became obese in adulthood (Hales & Barker 2001).

1.3 Potential mechanisms for the early life origins of disease

1.3.1 A genetic explanation for programming?

We have already introduced the 'Foetal Insulin Hypothesis' as one potential genetic mechanism linking low birth weight and later diabetes. Following studies, mainly by Davey Smith *et al* have added an extra twist to this hypothesis. They have illustrated a relationship between offspring of low birth weight and an increased risk of diabetes and cardiovascular disease in their parents (Davey *et al.* 1997; Lawlor *et al.* 2002; Smith *et al.* 2001; Smith *et al.* 2000). As discussed previously, the relationship between low birth weight and later risk of disease in adulthood may be explained by the programming effect of the intrauterine environment, but the relation between a baby's low birth weight and its parents' risk must have a different explanation; could a common genetic factor link this transgenerational association of birth weight and cardiovascular disease? Could the Foetal Insulin Hypothesis be correct? Lawlor *et al* are the first group to provide support for Hattersley and Tooke's hypothesis, by demonstrating an inverse relationship between the first-born birth weight and mothers' insulin resistance in late adulthood (Lawlor *et al.* 2002). Naturally, definitive support for the hypothesis would require the identification of genes or specific polymorphisms that are associated with low birth weight and diabetes or cardiovascular disease. Recent explorations have begun to scratch at this frontier, revealing some potential candidates (Casteels *et al.* 1999; Hattersley *et al.* 1998), though they still fall short of fully explaining the association (Ong *et al.* 2000; Hattersley *et al.* 1998). In the interim, Lawlors' study indicates that at least some of the association between low birth weight and disease may be genetic, or as in the case of 'lifestyle' risks, additive to the effects of an adverse intrauterine environment.

1.3.2 The environmental case for programming?

Stronger evidence suggests that it is the maternal environment, rather than genetic factors, that is the dominant influence on birth size. This concept was first aired in the 1950s when it was demonstrated in half-sibling studies that there was a stronger correlation between the birth weights of half-siblings sharing the same mother, than those sharing the same father (Morton 1955). Most interestingly, recent studies explain these findings as a conflict between

maternal and paternal alleles. In simple terms, paternally expressed imprinted genes, such as *Igf2*, promote foetal growth, thereby commandeering as many maternal resources as possible; whereas, maternally expressed genes inhibit foetal or placental growth thereby limiting the resources allocated to any one conceptus (Tycko & Efstratiadis 2002; Constancia *et al.* 2002). The importance of the maternal environment is further supported by embryo transfer experiments; a foetus transferred to a lager uterus will attain a larger birth size (Snow 1989). In monozygotic twin studies, the smaller one at birth has higher blood pressure (Levine *et al.* 1994) and the greatest risk of Type 2 diabetes (Poulsen *et al.* 1997). As these twins share the same genetic background, the differences in birth weight must be independent of genetic factors. In fact, more than 60% of the variation in birth weight between individuals is attributed to environmental factors (Penrose 1954). Moreover, the occurrence of associations between early life environmental manipulations and later physiology and disease risk in isogenic rodent models strongly implicates environmental factors (Holness & Sugden 2001; Seckl 2001). Finally, postnatal 'catch-up' growth is also a risk factor for subsequent hypertension (Barker *et al.* 1989b; Levine *et al.* 1994), ischaemic heart disease (Osmond *et al.* 1993) and insulin resistance (Leon *et al.* 1996), implying that smallness at birth due to intrauterine environmental growth restriction, rather than genetic smallness, is more important in determining the risk of adult disease.

1.3.3 Programming; the role of nutrition

Whilst maternal malnutrition during pregnancy is a popular explanation for the association of low birth weight and subsequent cardiovascular disease, the key evidence includes "severe" animal experiments, as few human studies verify the assertion.

The majority of the animal studies have utilised the rat, guinea pig, or sheep, and typically involve either restriction of food availability (Woodall *et al.* 1996; Persson & Jansson 1992); protein intake (Hales 1997) or availability (Whorwood *et al.* 2001); and sodium intake (Battista *et al.* 2002). In general terms, these models tend to mirror the profile of human *in utero* growth restriction. Thus in line with the 'Thrifty phenotype hypothesis' (Hales & Barker 2001), the undernourished foetus makes physiological adaptations from which it benefits in the short term by increasing substrate availability; but these changes become permanently programmed and ultimately counterproductive in post-natal life when nutrients

are readily available. When rats are fed a low protein diet (reduced by 55-67%), offspring have reduced birth weight, undergo catch-up growth (Vehaskari *et al.* 2001; Ozanne 2001) and develop insulin resistance (Ozanne & Hales 2002) and hypertension in adulthood (Langley-Evans *et al.* 1996). However, as we will later discuss, the characteristics of this common nutritional model may be mediated by more fundamental mechanisms, including foetal glucocorticoid overexposure.

As mentioned, there is rather sparse human evidence for the influence of maternal nutrition on the early life origins of disease. One study of Dutch adults conceived or born during late 1944 and early 1945, when the Nazi army halted food transport to occupied areas of the Netherlands, found that the offspring were of lower birth weight and developed glucose intolerance in adulthood (Ravelli *et al.* 1998). During this period food consumption plummeted from 1800 to 400 calories a day. Perhaps surprisingly, not every study of this time period revealed offspring of low birth weight (Roseboom *et al.* 2001). Furthermore, a cross-sectional study of people born during and after the extreme siege of Leningrad in World War II, failed to demonstrate an association between maternal undernutrition and subsequent diabetes mellitus or hypertension (Stanner *et al.* 1997). Finally, one prospective study examining maternal food intake showed no relationship between birth or placental weight, and any macro-nutrient (Mathews *et al.* 1999).

Clearly, severe malnutrition of various origins can programme adult disease in animals. However, the potential impact of subtle nutritional variations (as opposed to extreme starvation) of First World mothers on offspring health remains to be clarified.

1.3.4 Hormonal Programming

In response to undernutrition or prenatal stress, increases in the foetal levels of catabolic hormones (glucocorticoids, catecholamines, β-endorphins), and concurrent decreases in anabolic hormones (insulin, thyroxine, IGFs) help to modify foetal and placental metabolism, thereby maintaining fuel availability and foetal growth. However, many of these hormones have well documented life-long programming effects. This is particularly the case for steroids, illustrated by androgen's ability to programme adult levels of hepatic steroid metabolising enzymes, the development of sexually-dimorphic structures in the anterior hypothalamus as well as sexual behaviour (Gustafsson & Stenberg 1974b). These effects can only occur during a specific perinatal period, but then persist throughout life, largely

irrespective of any sex steroid manipulations. Similarly, several features of foetal glucocorticoid overexposure suggest a role in the early life programming of adult cardiovascular and metabolic disorders (Figure 1.1). This hypothesis is built on several pieces of evidence.

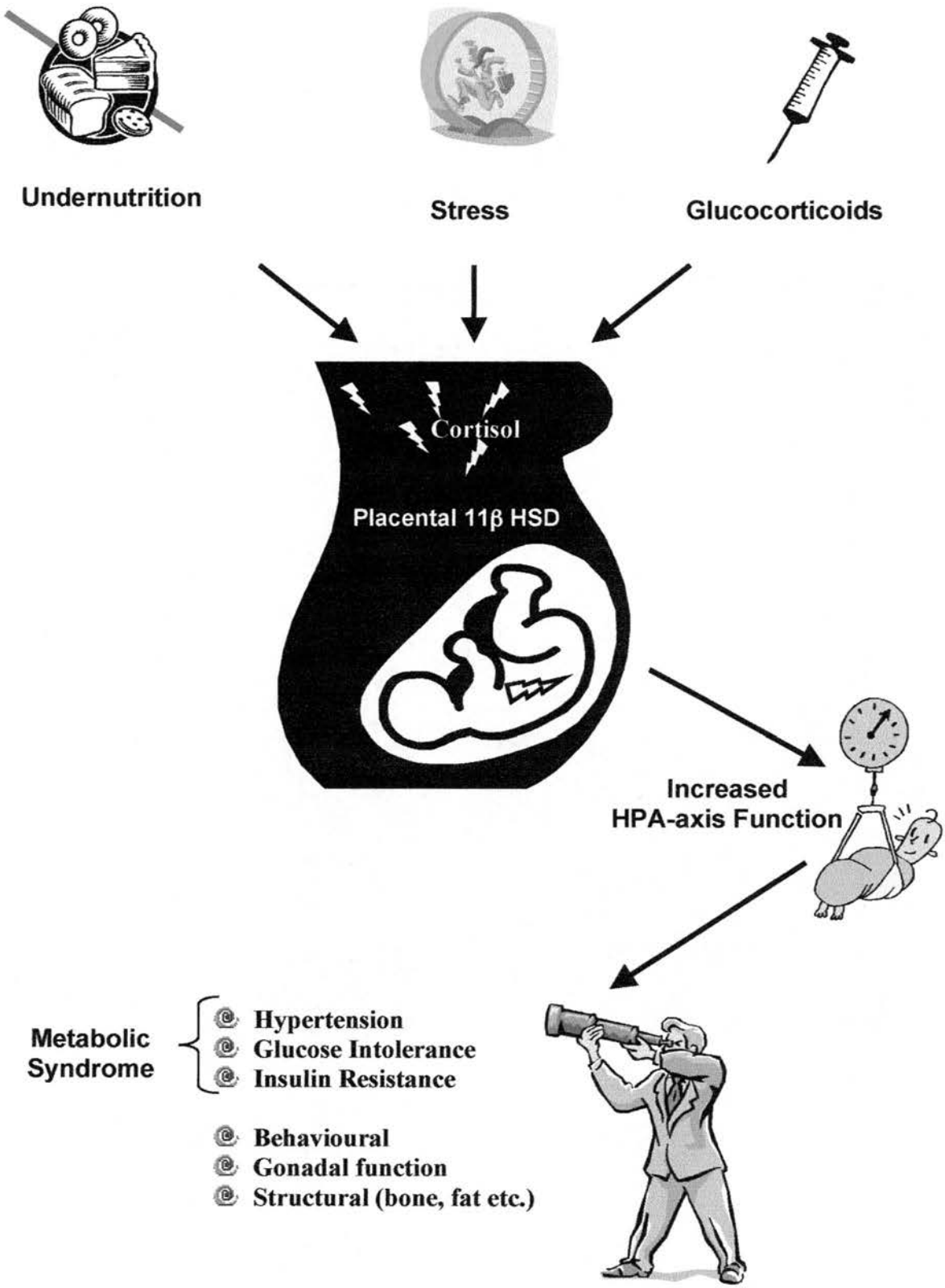


Figure 1.1 Programming of the Hypothalamic-Pituitary-Adrenal axis

1.4 Prenatal programming by glucocorticoids

1.4.1 Glucocorticoids and foetal growth

Life without glucocorticoids is eventually unsustainable. We have already reviewed their pivotal role in foetal life (section 1.2.1), and in the adult they are key players in the regulation of fluid and electrolyte homeostasis, blood pressure, the immune system, metabolism and physiological responses to stress. However, whilst some glucocorticoid is good, more is not necessarily better. Excessive foetal glucocorticoid exposure retards foetal growth and results in low birth weight offspring in humans as well as other primates and mammalian species (Reinisch *et al.* 1978; Novy & Walsh 1983). Foetal cortisol levels are increased in fetuses showing IUGR or in pre-eclampsia, implicating endogenous cortisol in retarded foetal growth (Goland *et al.* 1993). Placental 11β-hydroxysteroid dehydrogenase type II (11β-HSD-2) is responsible for maintaining lower foetal levels of circulating glucocorticoid (discussed further in section 1.4.2b), by inactivating endogenous hormone (Benediktsson *et al.* 1993). Activity of this enzyme correlates with birth weight. In rats, lower placental 11β-HSD-2 activity, and therefore greater foetal exposure to maternal glucocorticoids, is seen in the smallest fetuses (Benediktsson *et al.* 1993). Similar associations between birth weight and placental 11β-HSD-2 have been demonstrated in humans (Stewart *et al.* 1995), though not all studies have found this (Rogerson *et al.* 1997). Furthermore, humans homozygous for mutations of the 11β-HSD-2 gene, the rare 'syndrome of apparent mineralocorticoid excess', show greatly reduced birth weight compared to their unaffected sibs (Dave-Sharma *et al.* 1998). Finally, in pregnant rats, inhibition of the enzyme with licorice-derived drugs, such as carbenoxolone, reduces offspring birth weight (Lindsay *et al.* 1996a; Lindsay *et al.* 1996b). When viewed together, the above data provides compelling evidence for the importance of foetal glucocorticoid load during development.

The mechanisms by which glucocorticoids retard foetal growth are poorly understood, but most probably involve other hormones and growth factors, such as the insulin-like growth factors (IGFs). Glucocorticoids inhibit IGF production (Luo & Murphy 1989) and increase IGF binding protein (IGFBP) expression (Luo & Murphy 1990), thereby antagonizing IGF action and retarding foetal growth. Transgenic mice overexpressing IGFBP1 are of low birth weight and interestingly develop hyperglycaemia in adulthood (Rajkumar *et al.* 1995).

Together, these data promote the IGF system as one pathway through which prenatal glucocorticoids could affect both birth weight and metabolism.

There is also evidence that this pathway may underlie the actions of other programming agents, notably malnutrition. As mentioned (see section 1.3.3), severe dietary protein restriction during rat pregnancy produces offspring of low birth weight that subsequently develop hypertension in adulthood (Langley-Evans *et al.* 1996; Vehaskari *et al.* 2001). However, this model of programming is dependent on the actions of glucocorticoids. Furthermore, it has been shown that maternal undernutrition elevates cortisol levels (Fowden 1995) and suppresses foetal IGF-1 levels (Oliver *et al.* 1993). Interestingly, another study has shown that maternal protein restriction inhibits placental 11β-HSD-2 activity (Langley-Evans *et al.* 1996). Thus, a weakened placental barrier together with increased maternal cortisol production would significantly increase the foetal glucocorticoid load. Conclusively, in the rodent model, offspring may have their hypertension prevented by giving either the mother or her offspring inhibitors of glucocorticoid synthesis (Gardner *et al.* 1997; Langley-Evans 1997). Not only that, but the hypertension may be recreated by the concurrent replacement of corticosterone (Langley-Evans 1997), further emphasising the potential role of glucocorticoids as mediators of the effect of maternal malnutrition on foetal programming.

Thus, it is clearly evident that, glucocorticoids are central to mediating programming effects and that the foetal glucocorticoid load is a crucial factor in development. As to provide a more comprehensive understanding of the consequences of foetal glucocorticoid excess, we should firstly discuss the mechanisms of glucocorticoid action.

1.4.2 Mechanisms of glucocorticoid action

1.4.2a Synthesis, transport and metabolism

Glucocorticoids (cortisol in humans and other mammals, corticosterone in rodents) are synthesised in the mitochondria and endoplasmic reticulum of cells of the zona fasciculata/ reticularis in the adrenal cortex, under the regulation of the hypothalamic pituitary adrenal-axis (HPA-axis; Figure 1.2). Adrenocorticotrophin releasing hormone (ACTH), produced by corticotrophs in the anterior pituitary, binds to adrenal cortex cell surface receptors coupled to

adenyl cyclase. This rapidly activates the first, and rate limiting step in the synthesis of steroid hormones i.e. removal of the cholesterol side-chain by side-chain cleavage enzyme to form pregnenolone. Cortisol formation further requires the presence of endoplamic reticulum cytochrome p450-linked 17α-hydroxylase and 21-hydroxylase, and mitochondrial 11β-hydroxylase, which are only present in the adrenal zona fasciculate/ reticularis, thereby limiting glucocorticoid synthesis to this organ (Figure 1.3).

ACTH is regulated by CRH and arginine vasopressin (AVP). In basal states, levels of CRH, AVP and ACTH and cortisol are released in a pulsatile and circadian fashion, regulated by the suprachiasmatic nucleus of the hypothalamus. In man and other diurnal animals, peak secretion occurs in the early morning, while the nadir is usually around midnight, while in rodents, the cycle is reversed, reflecting their nocturnal activities. Glucocorticoid secretion is auto-regulated by a negative feedback loop, whereby glucocorticoids regulate the secretion of CRH and ACTH by binding to receptors in the hippocampus, hypothalamus and pituitary (Genuth 1992a).

Once secreted, glucocorticoids are predominantly protein bound to transcortin/ corticosteroid binding globulin (CBG; 70-75%) and albumin (15-20%), whilst only 5-10% circulates as free steroid (Genuth 1992b). Secreted molecules become bound to a receptor, or are degraded mainly within the liver and have a half-life of ~ 90 minutes. Metabolism is performed by a variety of enzymes including 5α and β reductases and 11β HSD-2. Conjugation of products in the liver by glucuronidation or sulphatation precedes excretion through the bile (mainly rodents) or kidney (mainly humans).

1.4.2b Pre-receptor modulation; 11β-hydroxysteroid dehydrogenase enzymes

Access to steroid receptors is regulated by the local activity of the 11β-HSDs, which are found in two isoforms. 11β-HSD type I is NADPH-dependent and is a predominant reductase, converting cortisone to cortisol in man, and 11-dehydrocorticosterone to corticosterone in rodents. Conversely, 11β-HSD-type II is NAD-dependent, and operates in the opposite direction i.e. type II enzyme is responsible for the de-activation, and type I enzyme the re-activation of glucocorticoids (Seckl & Chapman 1997).

11β-HSD-type1 is abundantly expressed in the liver and adipose tissue, where it acts to amplify the effect of glucocorticoids (Chapman *et al.* 1997). Its expression is under the regulation of glucocorticoids (Jamieson *et al.* 1999) and other hormones (Gomez-Sanchez *et al.* 2003), and its absence diminishes glucocorticoid-inducible responses (Kotelevtsev *et al.* 1997).

11β-HSD type II is expressed in sites also expressing the mineralocorticoid receptor (MR), including the kidney and placenta (Krozowski *et al.* 1995). This enzyme prevents exposure of MR to active glucocorticoids, which act as a ligand for MR, and circulate in higher concentration than aldosterone. Absence, mutation or inhibition of this isoform results in mineralocorticoid mediated salt retention and hypertension (Krozowski *et al.* 1999; Tomlinson & Stewart 2001; Stewart 1999; Kotelevtsev *et al.* 1999). Placental 11β-HSD type II is responsible for protecting the foetal HPA-axis from the much greater maternal levels of circulating glucocorticoids (Benediktsson *et al.* 1993). The implications of this are discussed in sections 1.4.1 and 1.5.

1.4.2c Glucocorticoid receptors; transcriptional activation and regulation of expression

Receptors for glucocorticoids are members of the steroid-thyroid-retinoid receptor super-family. Two cytoplasmic receptors bind glucocorticoids with differing affinity; the lower affinity Type 2 or glucocorticoid receptor (GR) is widely distributed in the brain and periphery, whereas the Type 1 receptor or mineralocorticoid receptor (MR) has a high affinity for glucocorticoids but is more restricted in anatomical localisation e.g. to the distal nephron, colon, hippocampus and sweat glands (reviewed by Bamberger *et al.* 1996; Funder 1997a; DeRijk *et al.* 2002).

Recently, an alternate splice variant of GR (or GRα), called GRβ, has been identified. However, the importance of this receptor isoform remains to be established (Bamberger & Chrousos 1995; Hecht *et al.* 1997), and given its absence in rodents (Otto *et al.* 1997), is somewhat controversial. Furthermore, in human cell lines, the presence of an alternately translated form of GRα has been found, resulting in further subdivision of human GRα into types A and B, with the latter being twice as efficient at trans-activation (Yudt & Cidlowski 2001; de Castro *et al.* 1996).

As glucocorticoids are fat-soluble, they enter cells by simple diffusion and activate the cytoplasmic receptor via a ligand-binding domain (Figure 1.2). This process involves dissociation from the heat-shock proteins (HSPs) that form a complex with unbound receptor (Bronnegard *et al.* 1995), and translocation of the hormone ligand complex across the nuclear membrane (Hache *et al.* 1999). Once its nuclear effect has been achieved, the receptor is recycled to the cytopolasm, where it re-associates with HSPs (Yang & Defranco 1996).

Ligand receptor classically exerts an effect through dimerisation and attachment of the DNA binding domain to specific sequences of DNA in the promoter regions of target genes, known as glucocorticoid response elements (GREs) (Reichardt & Schutz 1998). It can further achieve an effect by protein-protein interactions with other transcription factors, which bind DNA (Reichardt & Schutz 1998). Binding of GR and other transcription factors initiates chromatin remodeling and modulates the assembly of the transcriptional machinery, such that target gene transcription is activated or repressed (Reichardt & Schutz 1998). GREs have been identified in a variety of genes related to cardiovascular (e.g. angiotensinogen; Feldmer *et al.* 1991) and metabolic (e.g. PEPCK; Hanson & Reshef 1997) physiology.

As well as regulating the transcription from other genes, the expression of GR itself is regulated, mainly through the binding of transcription factors to response elements within its own promoter region, and also through post-translational mechanisms (Dong *et al.* 1988).

A further mechanism of GR regulation could be through the usage of alternate promoter regions of the gene (McCormick *et al.* 2000). The GR gene consists of 9 exons, the untranslated first containing the promoter region, and the remaining translated or protein encoding exons 2-9 (Funder 1997b). Multiple transcription initiation sites have now been identified within exon 1 of rats (McCormick *et al.* 2000), mice (Cole *et al.* 1993) and humans (Breslin *et al.* 2001) that yield transcripts with different 5' untranslated sequences, permitting transcriptional control through different specific response elements within the promoter region. This permits considerable complexity of the tissue (Kalinyak *et al.* 1989), age (Tohgi *et al.* 1995), sex (Owen & Matthews 2003), and metabolic (Burnstein *et al.* 1991) specific variation of control of GR expression and, potentially, programming (McCormick *et al.* 2000; O'Regan *et al.* 2001).

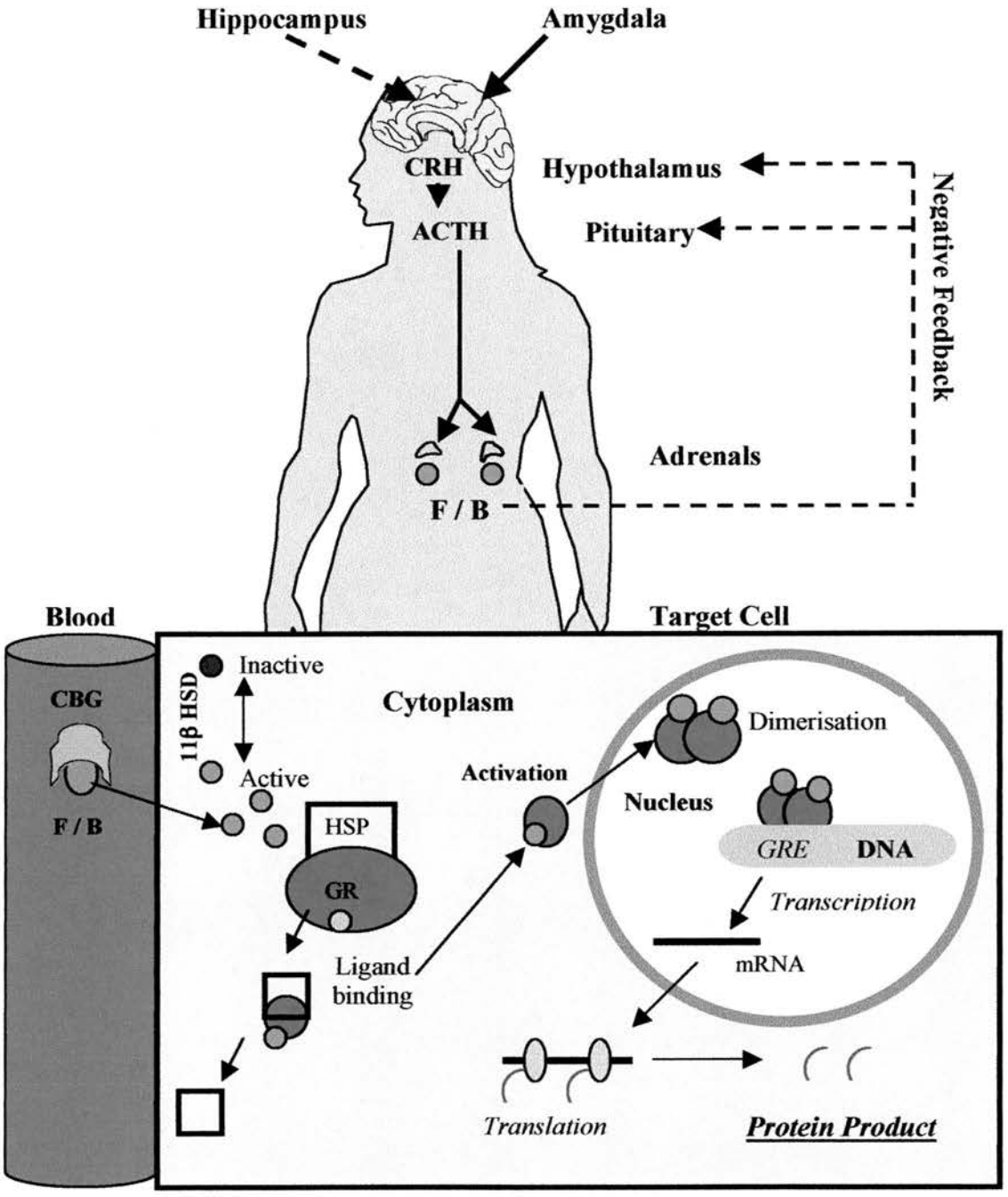


Figure 1.2 The Hypothalamic-Pituitary-Adrenal axis and model of glucocorticoid activity

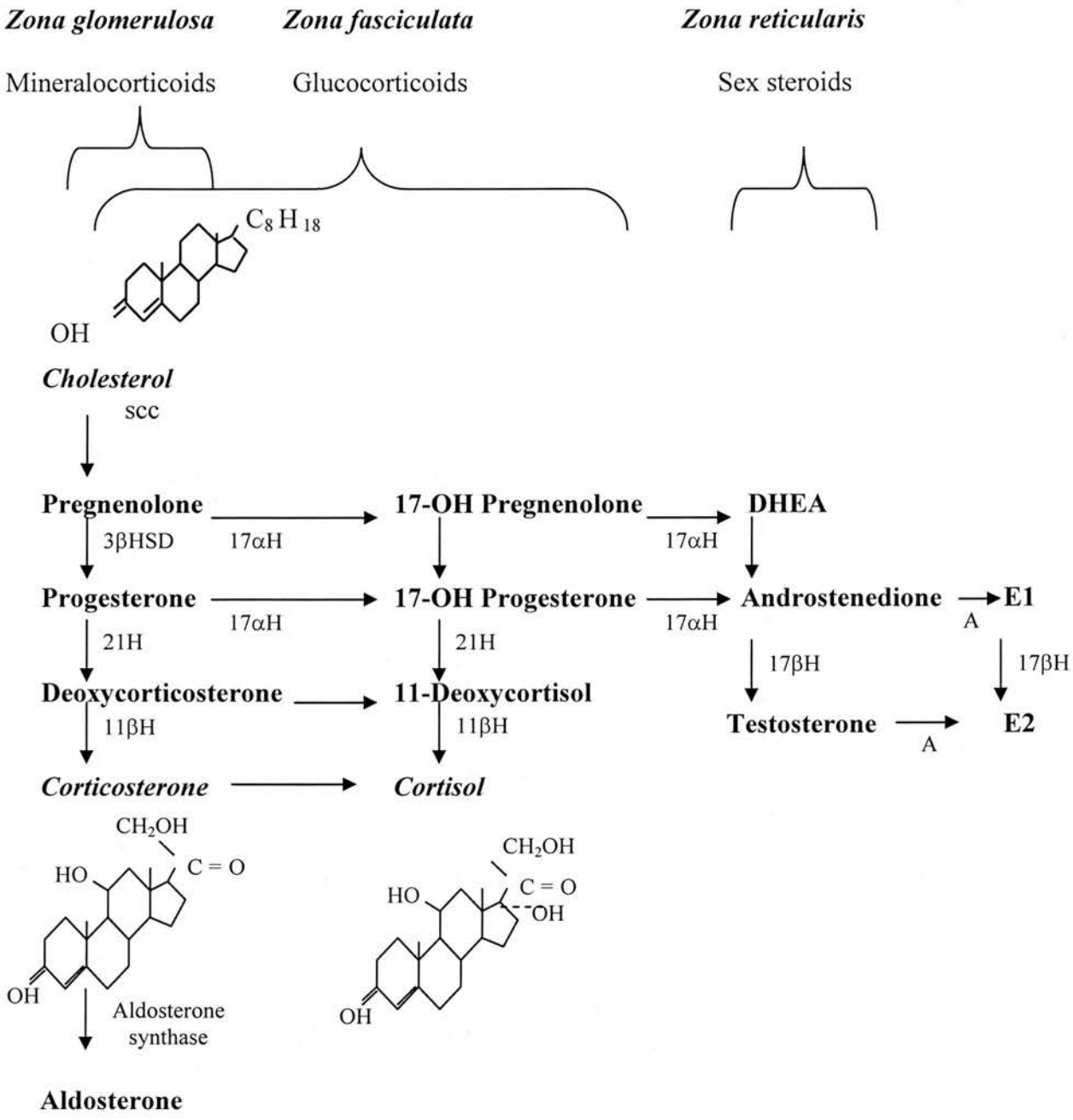


Figure 1.3 Summary of steroid synthetic pathway of the adrenal cortex

Cortisol and corticosterone are synthesised from cholesterol in the endoplasmic reticulum and mitochondria in the zona reticularis and zona fasciculata of the adrenal cortex. Aldosterone production occurs exclusively in the zona glomerulsoa.

Abbreviations: SCC: side cleavage chain, 17αH: 17α-hydroxylase, 21H: 21-hydroxylase, 11βH: 11β-hydroxylase, 3βHSD: 3β-hydroxysteroid dehydrogenase, 17βHSD: 17β-hydroxysteroid dehydrogenase, A: aromatase, E1: oestrone, E2; oestradiol.

1.4.2d Glucocorticoid induced insulin resistance and diabetes mellitus

Diabetes mellitus is a series of diseases classified by a lack of control over glycaemia. Type I diabetes is characterised by early onset and an inability of the pancreatic β-cells to secrete sufficient insulin, whereas, Type II or non insulin-dependent diabetes mellitus (NIDDM) has a typical onset in middle-age, and is related to poor glucose tolerance. Most patients with the latter form of diabetes are resistant to insulin-stimulated glucose uptake, as are ~ 25% of non-obese individuals with normal oral glucose tolerance, and require their pancreatic β-cells to sustain a permanent state of hyperinsulinaemia in order to prevent deterioration of glucose intolerance (Reavan 2000).

Insulin resistance reflects a reduced capacity for many tissues to respond to insulin (sometime referred to as the 'Insulin resistance syndrome'). In this scenario, hepatic gluconeogenesis is increased, whilst peripheral glucose uptake is significantly decreased (Boden 2001; Abel *et al.* 2001).

There are myriad potential sites of action for glucocorticoids to affect insulin sensitivity (reviewed by Andrews & Walker 1999). The principle effects of glucocorticoids are broadly antagonistic to those of insulin in the regulation of carbohydrate, lipid and protein metabolism by effects on three major sensitive tissues of liver, skeletal muscle and fat. Glucocorticoids increase blood glucose by mobilising substrates for hepatic gluconeogenesis. They stimulate the release of amino acids from skeletal muscle, fatty acids and glycerol from adipose tissue, and increase the expression of gluconeogenic enzymes such as PEPCK (Hanson & Reshef 1997), thereby enhancing gluconeogenesis in the liver. They also stimulate glycogen synthesis by activating glycogen synthase and inactivating the glycogen-mobilising enzyme glycogen phosporylase. Peripheral glucose uptake and utilisation is further inhibited by glucocorticoids, by decreased translocation of the GLUT 4 glucose transporter to the cell surface (Weinstein *et al.* 1998). Glucocorticoids additionally prevent glucose-stimulated insulin secretion from pancreatic β-cells (Delaunay *et al.* 1997). Moreover, given the adverse effects of glucocorticoids on endothelium-dependent vasodilatation (see section 1.4.2e); it is conceivable that the resulting altered blood flow would reduce glucose delivery to skeletal muscle.

Therefore, by increasing gluconeogenesis and inhibiting peripheral glucose uptake and insulin secretion, glucocorticoids are in a prime position to produce both insulin resistance and NIDDM. Unsurprisingly then, that clinical cases of cortisol excess or deficiency are associated with the most pronounced glucocorticoid mediated effects on glycaemic homeostasis. In cortisol deficiency, caused by hypopituitarism or hypoadrenalism (Addison's disease), tissue sensitivity to insulin is increased. Conversely, in cortisol excess (Cushing's syndrome); patients develop glucose intolerance, as well as hypertension and central obesity (referred together as the 'Metabolic Syndrome' or 'Syndrome X'). More interestingly though, it has been proposed that lesser perturbations within the HPA-axis, that push cortisol levels to the upper limit of normal, may contribute to the pathology of the Metabolic Syndrome (Bjorntorp & Rosmond 1999), a concept we'll elaborate on later.

1.4.2e Glucocorticoid induced hypertension and cardiovascular disease

In most Westernised countries, cardiovascular disease is the leading cause (almost 50%) of death in adults, as well as one of the leading reasons for major morbidity, social disruption and loss of income (Whelton 1984). In the United States alone, nearly 1 million people (or more than twice the population of Edinburgh) die each year from cardiovascular related disease. Three times as many suffer a serious clinical event and many more suffer unrecognized complications, such as silent myocardial infarction, which increase the individual's risk of subsequent morbidity and mortality. Hypertension is not only one of the most important predictors of cardiovascular disease but is also a major modifiable risk factor (Stamler *et al.* 1986). As with insulin resistance and NIDDM, glucocorticoids play a pivotal role in the regulation of blood pressure.

Blood pressure (BP) sensitivity to glucocorticoids is present from before birth in both humans (Kari *et al.* 1994) and other mammals (Berry *et al.* 1997), and hypertension is a common feature associated with the hypercortisolism of Cushing's syndrome (Sasano *et al.* 2002). Rats are particularly sensitive to glucocorticoids, with very low doses of dexamethasone (DEX) (a synthetic glucocorticoid commonly used in clinical practice) increasing blood pressure within a few days (Tonolo *et al.* 1988). The cardiovascular effects are associated with a marked reduction in body weight gain and even weight loss at supraphysiological doses. Short-term treatment of normal human volunteers with cortisol produces an effect akin

to that of mineralocorticoid excess, which differs markedly from the iatrogenic effects of synthetic steroids or the clinical consequences of Cushing's syndrome (Connell *et al.* 1987).

Glucocorticoid induced-hypertension is most probably mediated by alterations in: renal physiology, vascular reactivity, the bio-synthesis of vasoactive hormones, the renin-angiotensin-system (RAS) and the sympathetic nervous system (SNS) (Figure 1.4).

Glucocorticoids affect renal function by two main processes. DEX (which is a poor substrate for 11β-HSD type II, but is a greater GR than MR agonist), at concentrations which are unlikely to evoke a mineralocorticoid response, have been shown to cause a kaliuresis and to affect cation transport across the colon (Nickerson *et al.* 1969). The significance to BP control of this glucocorticoid response, which is presumably GR mediated, remains to be elucidated. Additionally, as we have previously discussed, if 11β-HSD type II is congenitally absent or inhibited pharmacologically, then physiological glucocorticoids are free to access renal MR causing sodium retention and hypertension.

Glucocorticoids also directly alter vascular responsiveness to circulating vasoactive agents (e.g. to noradrenaline (Russo *et al.* 1990) and angiotensin II (Sato *et al.* 1994; Grunfield 1990). For example, mesenteric arteries from DEX-treated rats are more sensitive to the effects of noradrenaline compared to controls (Russo *et al.* 1990). Furthermore, inhibition of nitric oxide synthesis (Wallerath *et al.* 1999) and decreased levels of both prostaglandin (a powerful paracrine vasodilator) and its mediator adenylate cyclase (Handa *et al.* 1984; Handa *et al.* 1983) have been demonstrated in adult rats with DEX-induced hypertension. Glucocorticoids also decrease the biosynthesis of atrial natriuretic peptide (ANP) (Whitworth *et al.* 1995), which has vasodilator and natriuretic properties, thereby contributing to their pressor effects. There is also evidence for inhibition of components of the vasodilatory kallikrein-kinin-system (Mantero & Boscaro 1992).

The RAS is a key regulator of BP (reviewed in Lavoie & Sigmund 2003; Jan Danser 2003). In adult rats, glucocorticoids regulate all of the principle components of the RAS including renin secretion (Burris *et al.* 1986), angiotensinogen synthesis and expression (Bunnemann *et al.* 1993), angiotensin converting enzyme activity (Mendelsohn *et al.* 1982), angiotensin II

(Sato *et al.* 1994) and mineralocorticoid receptor expression (Reul *et al.* 1989). Their greatest effect on the RAS is to increase the hepatic production of angiotensinogen (Mantero & Boscaro 1992). Furthermore, in the fetal rat, hepatic angiotensinogen expression is also regulated by glucocorticoids (Everett *et al.* 1991).

Increased sympathetic nerve activity has also been proposed as a mechanism to explain the pathogenesis of glucocorticoid-induced hypertension. This could arise from changes in neuronal or extraneuronal uptake of noradrenaline. Decreased neuronal uptake normally potentiates vasoconstriction caused by catecholamines (Dusting & Li 1986).

Additionally, glucocorticoids have also been shown to directly increase cardiac output in man (Pirpiris *et al.* 1993), and to promote sodium influx into vascular smooth muscle cells (Kornel *et al.* 1993).

Indirectly, glucocorticoid-induced hyperinsulinaemia may itself promote hypertension. Insulin resistance is a common feature of essential hypertension (DeFronzo 1992), and several lines of evidence suggest that insulin resistance per se or hyperinsulinaemia can result in increased sodium reabsorbtion (DeFronzo *et al.* 1975; DeFronzo 1981), enhanced vascular tone and activate the adrenergic nervous system (DeQuattro & Feng 2002), thereby driving hypertension.

In summary, glucocorticoids potentiate the pressor activity of vasoconstrictors, increase the activity of the RAS and SNS, and reduce the activity of a number of potent vasodilators. Furthermore, glucocorticoid-induced hyperinsulinaemia may provide an additional mechanism promoting hypertension.

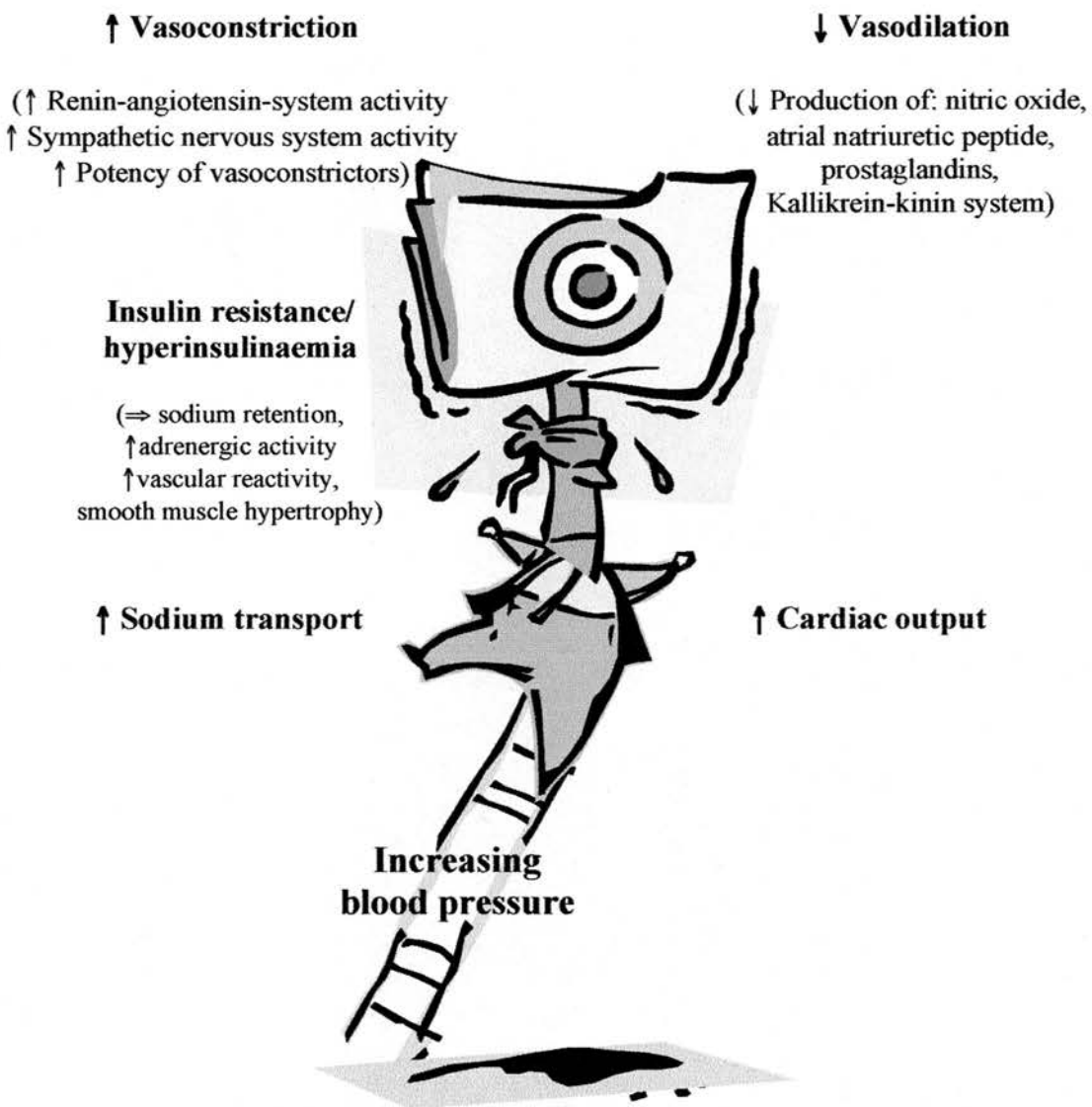


Figure 1.4 Mechanisms of glucocorticoid-induced hypertension

1.4.2f Role of glucocorticoids in The Metabolic Syndrome

As briefly mentioned, the 'metabolic syndrome' (or 'syndrome X') is a cluster of abnormalities that are risk factors for the development of ischaemic cardiovascular disease (Reaven 2000; Reaven 2002). These include insulin resistance with compensatory hyperinsulinaemia, glucose intolerance, increased plasma triglycerides, decreased high-density lipoprotein cholesterol, hypertension (Reaven 2002), and frequently obesity (Bjorntorp & Rosmond 1999).

We have previously discussed how glucocorticoids can impact individually on each of these variables, but more recent studies have focused on the influences of cortisol secretion on these collective cardiovascular risk factors. In cross-sectional and case-control studies, higher blood pressure, glucose intolerance, insulin resistance and hyperlipidaemia are associated with elevated cortisol levels, measured in blood, saliva and urine (Phillips *et al.* 2000; Fraser *et al.* 1999; Walker *et al.* 1998; Phillips *et al.* 1998; Stolk *et al.* 1996; Filipovsky *et al.* 1996) or impaired peripheral inactivation of cortisol (Walker *et al.* 1998).

Tissue sensitivity to glucocorticoids is partially mediated by the density of GR expression, and can be assessed clinically by measuring the intensity of dermal blanching after topical administration of DEX. Individuals with cardiovascular risk factors reveal altered peripheral glucocorticoid sensitivity. Thus, increased vasoconstrictor responses are found in men with glucose intolerance and insulin resistance (Walker *et al.* 1998), essential hypertension (Walker *et al.* 1996) or with a familial predisposition to hypertension (Walker *et al.* 1998). This response is also enhanced in healthy individuals with GR gene polymorphisms (Panarelli *et al.* 1998), also associated with familial predisposition to hypertension (Watt *et al.* 1992) and greater hyperinsulinaemia (Weaver 2001).

Tissue sensitivity to glucocorticoids may also be adjusted by the 11β-HSDs (see section 1.4.2b), and several strands of evidence suggest that 11β-HSD type I plays a role in coordinating metabolic control (reviewed in Seckl and Walker 2001). Recent studies suggest that impaired 11β-HSD type I activity contributes to the pathogenesis of obesity, a feature of the metabolic syndrome. In the leptin-resistant Zucker obese rat, 11β-HSD type I activity is impaired in the liver, a change predicted to ameliorate the local intrahepatic metabolic

consequences of the obesity (Livingstone *et al.* 2000). However, this may also activate the HPA axis to compensate for the increased clearance of glucocorticoids through reduced hepatic regeneration. Furthermore, tissue-specific enhanced 11β-HSD type I activity may also promote adverse metabolic effects. For example, whilst Zucker obese rats have impaired hepatic 11β-HSD type I activity, they show enhanced activity of 11β-HSD type I in their omental adipose tissue (Livingstone *et al.* 2000). Interestingly, this very same pattern of 11β-HSD type I dysregulation (i.e. impaired in liver, enhanced in adipose) has been demonstrated in human obesity (Rask *et al.* 2001).

Clearly this evidence suggests that altered HPA-axis activity i.e. increased circulating levels of glucocorticoids together with increased GR sensitivity, contributes to the pathogenesis of insulin resistance and the development of cardiovascular risk factors. Given the wealth of evidence from epidemiological studies highlighting the association between low birth weight and the development of these cardiovascular risk factors; it hardly surprising that glucocorticoids have been proposed to link low birth weight and the predisposition to disease in adult life (Edwards *et al.* 1993; Benediktsson *et al.* 1993; Seckl *et al.* 1999). In fact the 'low birth weight syndrome', resembles an attenuated metabolic or Cushing's syndrome phenotype (diabetes, hypertension, heart disease). This hypothesis continues to be endorsed by an ever-growing number of both animal and human studies, reviewed below.

1.5 Adult phenotype of prenatal glucocorticoid programming

Several groups have begun to examine the effect of prenatal glucocorticoid exposure on long-term health and metabolism, considering glucocorticoid sensitive physiological systems.

In rats, prenatal exposure to excess exogenous (dexamethasone; DEX) or endogenous (inhibition of 11β-HSD type II by carbenoxolone) glucocorticoids (Figure 1.5) results in offspring of low birth weight (by 10-16%) (Nyirenda & Seckl 1998; Welberg *et al.* 2001a; Levitt *et al.* 1996; Sugden *et al.* 2001), that in adulthood have elevated circulating basal corticosterone levels (Levitt *et al.* 1996; Welberg *et al.* 2001a), develop hypertension (Benediktsson *et al.* 1993), and glucose intolerance and insulin resistance (Nyirenda & Seckl 1998; Nyirenda *et al.* 2001), i.e. all the major components of the metabolic syndrome. Adverse effects of treatment have further been observed in the kidney (Ortiz *et al.* 2003),

heart (Langdown *et al.* 2001), immune system (Bakker *et al.* 1998) and brain (Welberg *et al.* 2001a), which will be discussed further in the next section.

Similar effects of prenatal glucocorticoid treatment have also been noted in guinea pigs (Liu *et al.* 2001) and the sheep; where repeated DEX treatment in early pregnancy programmes hypertension (Dodic *et al.* 1998), and in mid to late gestation produces glucose intolerance (Gatford *et al.* 2000).

The existence of glucocorticoid programming in humans is just beginning to be appreciated. High-dose glucocorticoid treatment during human pregnancy reduced birth weight (Reinisch *et al.* 1978). In rhesus monkeys, brief high doses of DEX in the last trimester affects hippocampal structure and cortisol levels in the offspring (Uno *et al.* 1994). Clinical evidence concerning the effects of low-dose *in utero* glucocorticoids is rather sparse. Substrates for 11β-HSD type II (cortisol and prednisolone) would be anticipated to have little effect. Conversely, synthetic, non-11β-HSD type II substrate glucocorticoids are used in obstretics for two distinct indications. DEX, betamethasone or triamcinolone are often given to accelerate foetal lung maturation (thereby preventing respiratory distress syndrome) when preterm labour threatens (Ward 1994). DEX is rarely administered throughout gestation, except in cases of congenital adrenal hyperplasia; DEX attenuates adrenal androgen excess, thereby reducing virilisation (Speiser 1999). In this scenario birth weight has been reported as normal (Forest *et al.* 1993), but as we've previously commented, birth weight is only a crude indicator of an adverse intrauterine environment. As children, these individuals display altered psychosocial behaviour being shyer and more emotional (Trautman *et al.* 1995), as well as demonstrating delayed psychomotor development (Lajic *et al.* 1998). More interestingly, late trimester DEX has been shown to be associated with a relative increase in BP in adolescence (Doyle *et al.* 1999). Later adult outcomes will undoubtedly be the subject of ongoing surveillance.

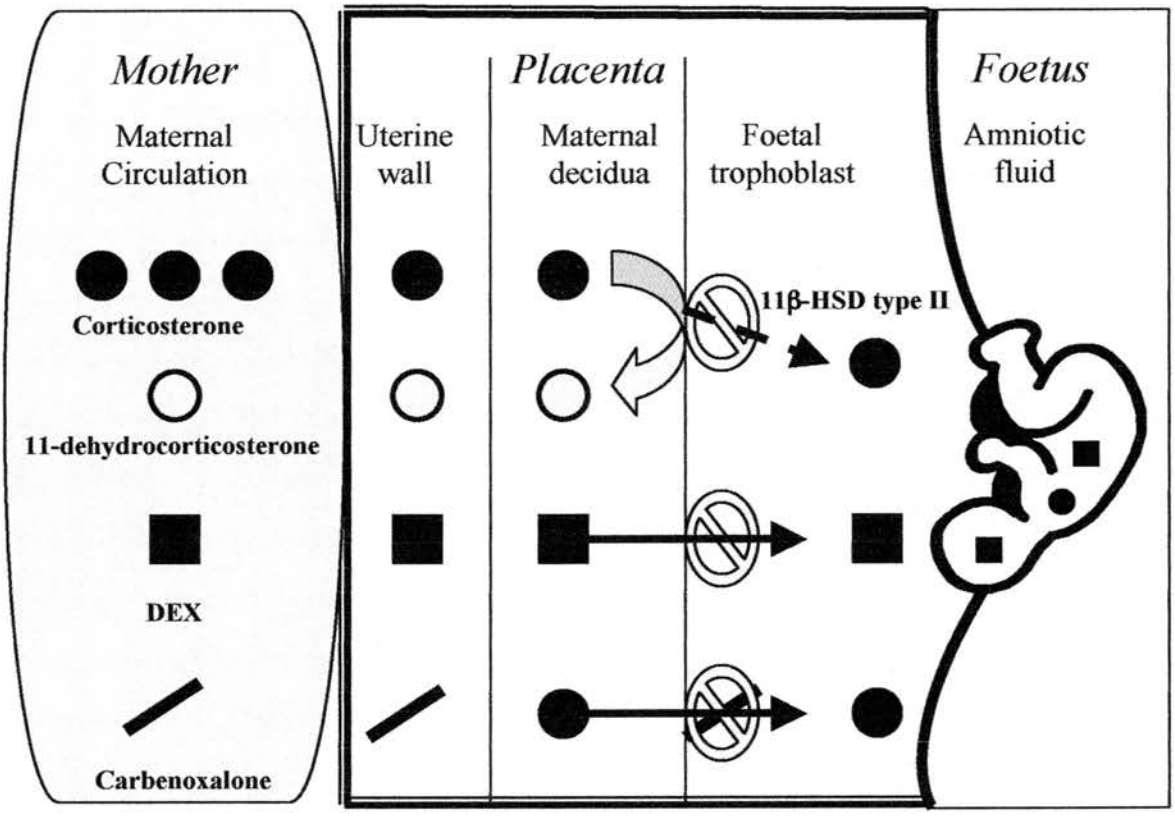


Figure 1.5 The placental barrier, and mechanisms of *in utero* glucocorticoid over-exposure

Foetal levels of corticosterone are much less than the maternal circulating levels due to the enzyme 11β-hydroxysteroid dehydrogenase-type II (11β-HSD type II), which inactivates the majority of maternal glucocorticoid diffusing across the placenta. Foetal over-exposure to glucocorticoids may occur if dexamethasone (DEX), which is a poor substrate for the enzyme, is administered to the mother, ***or*** if carbenoxolone, which inhibits the enzyme, is administered during pregnancy.

1.6 Physiological mechanisms of prenatal glucocorticoid programming

1.6.1 HPA-axis programming

A series of studies have examined administration of ACTH or cortisol to pregnant rats. Since neither ACTH nor physiological corticosteroids freely pass the placenta, the results have been unsurprisingly modest and discordant, probably a reflection of the doses used than any consistent pattern of effects (reviewed by Welberg & Seckl 2001). In contrast, prenatal exposure to DEX, which readily passes the placenta, reduces brain weight (DeKosky *et al.* 1982) and alters adult behaviour (producing behavioural inhibition and reduced coping in adverse situations) (Welberg *et al.* 2001a), though the outcome depends upon the timing of gestational exposure. These effects may reflect altered amygdala gene expression and function with CRH, GR and MR genes implicated (Welberg *et al.* 2001a), so far in specific nuclei of this region underpins fear and learning as well as driving HPA activity.

Prenatal DEX also affects HPA-axis activity in the rat. Both long and short term exposure to DEX *in utero* result in adult rats with elevated basal glucocorticoid levels (Levitt *et al.* 1996; Welberg *et al.* 2001a). However, the mechanisms underlying the increased HPA-axis activity appears to differ depending upon the timing of exposure. DEX exposure in the final trimester of pregnancy increases CRH mRNA in the hypothalamic PVN and reduces hippocampal MR and GR (Welberg *et al.* 2001a), whereas DEX throughout gestation does not alter hippocampal GR or MR, but increases receptor expression in the amygdala, a structure which stimulates the HPA-axis (Levitt *et al.* 1996; Welberg *et al.* 2001a). Thus late gestational exposure to DEX may permanently alter the 'set point' of the HPA-axis at the level of the hippocampus, reducing feedback sensitivity, whereas continuous exposure to DEX throughout gestation may increase forward drive of the HPA-axis at the amygdala. Thus, distinct neural mechanisms appear to modulate the common outcome of altered HPA-axis activity following prenatal glucocorticoid exposure. Similar heterogeneity of effects has been emphasised by recent studies in the guinea pig (reviewed by O'Regan *et al.* 2001).

How might such mechanistically distinct effects come about with exposure during different times? As mentioned previously, it is intuitive that programming can only occur during the development of a tissue. Thus, glucocorticoid exposure in the last days of gestation in the rat can only target those actively developing CNS regions, such as the hippocampus, whilst those regions that are yet to develop or are already in their final state are protected. Of course the long and complex pre-and postnatal ontogeny of the brain make it a prime target for programming. Complex patterns of expression of the key candidate genes GR, MR and 11β-HSDs in the brain may underlie this (Diaz *et al.* 1998; Matthews 1998). Indeed, 11β-HSD type II is highly expressed in mid-gestation CNS and the outcome for the HPA-axis and behaviour of its inhibition during pregnancy appears to encompass some aspects of both early and late DEX exposure, consistent with this notion (Welberg *et al.* 2001a). Whilst the details of ontogenic patterns are species-specific (for example near term the guinea-pig shows a dramatic reduction in PVN GR (Matthews 1998), whereas the sheep shows reduced hippocampal GR (Andrews & Matthews 2000), perhaps both necessary to facilitate the glucocorticoid surge necessary for parturition in these species (Challis *et al.* 2001), the broad spectrum of tissues protected from or allowed timed exposure to glucocorticoids appears a tenable interpretation of these complex patterns of gene expression. Clearly glucocorticoids (whether endogenous or exogenous) can only have developmental effects on specific target genes and systems during their individual ontogenic windows of susceptibility.

The evidence for programming of the HPA-axis in humans has only begun to be investigated. Intriguingly, birth weight correlates closely with HPA-axis measures from infancy (Clark *et al.* 1996), through young adulthood (Levitt *et al.* 2001) to old age (Phillips *et al.* 1998; Phillips *et al.* 2000). Low birth weight associates with both increased basal and ACTH-stimulated cortisol levels (Levitt *et al.* 2001). Even though roles for MR and GR remain to be fully elucidated, evidence supporting a physiological role for MR in human central feedback includes the observation that MR antagonists potassium canrenoate and spironolactone both increase plasma cortisol (Heuser *et al.* 2000; Arvat *et al.* 2001). Whether prenatal exposure to DEX or liquorice derivatives have similar human HPA-axis programming effects is unknown, though fludrocortisone decreases plasma cortisol levels, hinting at impaired central negative feedback (Mangos *et al.* 2000) (of course positive HPA-axis drive could be equally important). All remain key issues to be explored in human cohorts.

1.6.2 Programming of insulin-glucose metabolism

In rats, prenatal DEX reduces adulthood glucose-induced insulin secretion (Holness & Sugden 2001), probably due to a programmed reduction in β-cell mass (Petrik *et al.* 1999), thereby causing impaired pancreatic function. Whilst the molecular changes underpinning this remain to be fully documented, recent studies suggest increases in pancreatic GR are at least partially responsible (Sugden & Bulmer 2001).

Permanent up-regulation in the activity of a key hepatic gluconeogenic enzyme, PEPCK, has also been reported (Nyirenda & Seckl 1998). This effect is confined to the gluconeogenic periportal region of the hepatic acinus, where strikingly, GR expression is also increased (Nyirenda & Seckl 1998). Thus, it is likely that hypercorticosteronaemia in these animals, act on higher hepatic levels of GR to increase PEPCK activity, thereby promoting increased gluconeogenesis. Moreover, increased GR and attenuated fatty acid uptake, specifically in visceral adipose (a key clinically important metabolic depot) is consistent with insulin resistance, and could contribute to the increased hepatic glucose output in this model (Cleasby *et al.* 2003). Finally, the window for these programmed effects is restricted to the last trimester (Nyirenda & Seckl 1998), and operates directly on the foetus / neonate, rather than via indirect effects on maternal postnatal nursing behaviour (Nyirenda *et al.* 2001).

1.6.3 Programming blood pressure regulation

Whilst much work has elucidated the mechanisms of programmed insulin/ glucose metabolism, relatively little is known of the mechanisms of DEX programmed hypertension. In rats, prenatal DEX, largely in the last trimester, affects the development and maturation of specific organs related to blood pressure control and maintenance. Specifically, prenatal glucocorticoids or stress affect the developing heart, vasculature, kidney and brain. Effects include permanent induction of the pattern and balance of alpha and beta adrenergic receptor expression (Huff *et al.* 1991) and potentiation of adenylate cyclase (Bian *et al.* 1993); either might alter the subsequent vascular responsivity to vasoconstrictors, though this awaits investigation. Development of the heart and its biochemistry are also programmed by prenatal DEX (Bian *et al.* 1993), as is the sympathetic innervation of some organs (Navarro *et al.* 1991; Kudlacz *et al.* 1990). These effects may be arterial bed specific, as with

endothelin-1 sensitivity (Docherty *et al.* 2001). Yet, to what extent these and other possible perturbations in local and/ or the systemic SNS mediate DEX programmed hypertension remains unknown.

Some data in sheep have implicated the renin-angiotensin-aldosterone system (RAS) (Moritz *et al.* 2002; Dodic *et al.* 2002). For example, foetal sheep directly infused with cortisol become hypertensive and show increased pressor responses to angiotensin II (Tangalakis *et al.* 1992). In the rat, it is unknown whether prenatal DEX-induced changes in RAS also occur, or if they have life long cardiovascular implications. As mentioned previously, glucocorticoids regulate all aspects of the RAS in the adult rat (see section 1.4.2e), and more importantly, in the foetal rat, they regulate hepatic angiotensinogen expression (Everett *et al.* 1991).

Finally, most blood pressure studies in the rat have utilised either tail-cuff plethysmography or carotid cannulation (Benediktsson *et al.* 1993; Levitt *et al.* 1996; Lindsay *et al.* 1996a). Both techniques are marred by serious technical artifacts (see Chapters 2 and 5 for further discussion); moreover, measurements are recorded during the daytime, when offspring are in their quiet phase of their circadian rhythm. Therefore, non-stressful and physiologically accurate 24 hour blood pressure and haemodynamic profiling is clearly required. Only then, will we be fully equipped to comprehend the true nature and real impact of prenatal glucocorticoid treatment on cardiovascular physiology.

1.6.4 Effect of perinatal stress on programming

Prenatal environments exert profound influences on the development of an organism and can predispose it to adaptive disturbances in later life. For example, maternal stress, particularly during the final trimester, has been shown to have long term programming effects on rat offspring (Peters 1982; Dunn & Berridge 1990). Interestingly, these effects are analogous to those seen in adult offspring of dams treated with DEX during the last week of pregnancy i.e.: hyperactivation of the HPA-axis (Hennessy *et al.* 1999), altered corticosterone secretion (Henry *et al.* 1994; Reul *et al.* 1994), altered GR expression (Koehl *et al.* 1999), increased tissue sensitivity to glucocorticoids (McCormick *et al.* 1995) and increased stress responsiveness (Takahashi *et al.* 1992). Given that a key feature of the stress response is the

secretion of glucocorticoids (Selye 1976), it is most probable that endogenous increases in foetal glucocorticoid exposure during stress are responsible for the similarities shared between the two prenatal models. Additionally, elevated maternal glucocorticoid levels may further modify glucocorticoid secretion in the offspring by acting on developing noradrenergic systems (Barbazanges *et al.* 1996). Indeed, prenatal stress increases the turnover of brain noradrenaline in adult rats (Takahashi *et al.* 1992)(as does prenatal DEX; Slotkin *et al.* 1992), and noradrenaline exerts a direct inhibitory control on hippocampal corticosteroid receptors, thus facilitating corticosterone secretion (Maccari *et al.* 1992 ; Yau & Seckl 1992).

Programming effects are not just restricted to prenatal manipulations, as 'neonatal handling' (Meaney *et al.* 1996 ;O'Donnell *et al.* 1994) and stress (Meaney *et al.* 1996) have also been shown to exert long-term effects on stress susceptibility. In the 'neonatal handling' paradigm, 15 minutes of daily 'handling' of pups during the first week or two of life permanently increases hippocampal GR, thereby potentiating the HPA-axis sensitivity to glucocorticoid negative feedback (Meaney *et al.* 1988); ensuring low levels of plasma glucocorticoids, a state compatible with good adjustment to environmental stress (Meaney *et al.* 1992). Neonatal glucocorticoid exposure appears to have similar effects (Catalani *et al.* 1993). Neonatal handling further enhances maternal care-related behaviours, which correlates with offspring HPA-axis physiology and hippocampal GR expression (Liu *et al.* 1997). Moreover, the long-term effects of some prenatal programming can be considerably modified in the very early postnatal period (Maccari *et al.* 1995).

Therefore, by operating through distinct developmental 'windows', similar early life events can produce manifold differing manifestations, that are wholly dependent on the degree, duration, developmental timing or sequence of events. This equally applies to programmed human pathophysiology (Seckl 2001). However, the long-term cardiovascular and metabolic consequences of any form of perinatal stress remains to be elucidated.

1.6.5 Effect of gender on programmed phenotypes

In several other paradigms, perinatal manipulations that permanently alter adult HPA-axis function are sex-specific (Weinstock *et al.* 1998; Mathews *et al.* 1999), and the importance of gender in determining programmed outcomes has recently been suggested in both humans

(Adair & Cole 2003) and rodents (Ortiz *et al.* 2003 ; Khan *et al.* 2003) . Moreover, there is no reason to assume that if a similar programmed phenotype is obtained that identical mechanism underlie it. Indeed, early or late gestation DEX or 11β-hydroxysteroid dehydrogenase inhibition all produce HPA axis hyperactivity but involve distinct central mechanisms (Welberg *et al.* 2001a; Welberg *et al.* 2001b; Welberg *et al.* 2000). However, little work has been done on the effects of prenatal steroids and peripheral programming phenotypes. Recently, gender-specific patterns of programmed insulin resistance have been observed in the male offspring of dams, whose diet was protein restricted during pregnancy and lactation (Sugden & Holness 2002). DEX-programmed hypertension occurs in both sexes, but may reflect distinct processes in each gender, whilst hyperglycaemia/hyperinsulinaemia has only been shown in male rats.

1.7 Hypothesis

Against this background, we hypothesised that prenatal DEX given in the last week of pregnancy would result in permanent alterations in both the RAS and SNS, which in adulthood would produce altered cardiovascular and metabolic phenotypes. We further hypothesised that these altered phenotypes would be gender specific.

1.8 Objectives and Aims

In examining our hypothesis we addressed the following key questions:

- What are the effects of perinatal stress upon the cardiovascular and metabolic phenotypes of *in utero* DEX and control treated rats?
- Are there gender differences in the prenatal glucocorticoid programming of cardiovascular and metabolic physiology?
- Does prenatal DEX treatment programme offspring RAS, and, if so, does this contribute to their cardiovascular phenotype?
- Do alterations in dietary sodium affect the metabolic and cardiovascular phenotypes of *in utero* DEX and control treated rats?
- What are the circadian blood pressure, haemodynamic and activity profiles of a rat, programmed by prenatal over-exposure to glucocorticoids?
- What are the roles of environmental stress and the SNS in determining programmed cardiovascular outcomes?

Materials & Methods

2.1 Materials

Materials were sourced from the following:

2.1.1 General Chemicals

Agar Bactotryptone Yeast Extract	*Becton Dickinson, Crowley, Oxon, UK.*
Acids Formamide Formaldehyde Other organic solvents	*BDH-Merck, Poole, Dorset, UK.*
Cryo-m-bed-embedding compound	*Brights, UK.*
Ethanol	*Hayman Ltd., Witham, Essex, UK.*
Malate Dehydrogenase	*Roche Diagnostics Ltd., Lewes, East Sussex, UK.*
All other solid chemicals	*Sigma-Aldritch Company Ltd., Poole, Dorset, UK.*

2.1.2 Molecular Biologicals

TaqMan™ primers and probes	
TaqMan™ PCR core reagent kit	*Applied Biosystems, Applera, Warrington, Cheshire, UK.*
NICK sephadex G-50 DNA column	*Amersham Pharmacia Biotech, Little Chalfont, Buckinghamshire, UK.*
Zeta-Probe® GT, Nylon membrane	*Bio-Rad Laboratories Ltd., Hemel Hempstead, Hertfordshire, UK.*
Agarose	*Biowhittaker Molecular Applications, Wokingham, Surrey, UK.*
PicoFluor 40 Scintillant fluid	*Canberra Packard, UK.*
NTB-2 photographic emulsion	
Kodak D19 developer	
Amfix high speed fixer	*H.A. West, Edinburgh, UK.*
1kb DNA Ladder	
First strand buffer	
Low Melting Point Agarose	
TRIzol®	*Invitrogen Life Technologies/Gibco BRL, Paisley, UK.*
Dialysis tubing	*Phillip Harris Scientific, Ashby-de-la Zouch, Leicestershire, UK.*

Restriction enzymes	
Nucleotides	
Transcription buffer	
DTT	
BSA solution	
RNA polymerases	
HB101 cells	
p-GEM-T Easy Vector cloning kit	
Taq DNA Polymerase and buffer	
Wizard Plus Miniprep DNA purification System	*Promega Ltd., Southhampton, Hants, UK.*
Qiagen PCR purification kit	*Qiagen Ltd., Crawley, West Sussex, UK.*
Random Primed DNA Labeling Kit	*Roche Products Ltd., Welwyn Garden City, Hertfordshire, UK.*
Phenol/Chloroform/Iso-amyl alcohol (25:24:1)	
Kodak Biomax MS film	
Kodak Biomax MR film	*Sigma.*
Oligonucleotides	*TAGN, Newcastle-Upon-Tyne, UK.*

2.1.3 Protein Reagents

Bradford Protein Assay Reagent	*Bio-Rad Laboratories Ltd.*

2.1.4 Antibodies

Anti-Angiotensin 1 antibody

Anti-Corticosterone antibody	*Generous gifts from Dr. C. J. Kenyon.*

2.1.5 Radioisotopes

[1,2,6,7-^{3}H] Corticosterone

2.85 TBq/mmol

[α-^{32}P]-GTP,

111TBq/mmol

^{125}I Angiotensin I

370KBq/mmol

^{35}S-UTP

30TBq/mmol

Amersham Pharmacia Biotech.

2.1.6 Animals

Wistar rats were purchased form *Harlan Orlac, Bicester, Oxon, UK.*

Standard chow was supplied by *Special Diet Services (SDS), Witham, Essex, UK*, and contained 61.9% carbohydrate, 18.8% protein, 3.4% oil and 0.6% salt.

Scientific Diet Service, a division of SDS supplied the Normal Sodium (0.3%), Low Sodium (0.03%) and High Sodium (3%) diets, which contained the same carbohydrate, protein and oil as standard chow.

2.1.7 Drugs

Saline (0.9% w/v)	*Antigen Pharmaceuticals Ltd., Roscrea, Ireland.*
Nubain (Nalbuphine Hydrochloride)	*Du Pont, UK.*

Halothane-VET	*Merial Animal Health Ltd., Dublin, Ireland.*
Sagatal (sodium pentobarbitone, 60mg/ml)	*Rhone Merieux, Essex, UK.*
Multiparin® (Heparin, 5000u/ml)	*Roche Products Ltd.*
Dexamethasone (±)-Arterenol ([±]-Norepinephrine [+]-hydrogen tartrate) [Arg^8] Vasopressin Acetate salt Reserpine D-amphetamine	*Sigma*

2.1.8 Materials for plasma assays

Anti-rabbit scintillation proximity assay reagent	*Amersham Pharmacia Biotech.*
Rat Insulin ELISA Kit (Containing Antibody-coated microplates, 2.56ng lyophilised rat insulin standard, sample dilutent, Anti-Rat Insulin Enzyme Conjugate stock solution, Enzyme conjugate dilutent, enzyme substrate (TMB) solution, enzyme reaction stopping solution (1N Sulphuric acid), X20 concentrated washing buffer stock solution)	*Crystal Chem Inc., Chicago, Illinois, USA.*
Count-A-Coat Aldosterone Radioimmunoassay	

(Containing 200 Aldosterone Ab-coated polypropylene tubes, lyophilised iodinated aldosterone, 8 lyophilised processed human aldosterone calibrators)

Diagnostic Products Corporation, LA, USA.

ACTH IMRA

(Containing 7 lyophilised ACTH calibrators in human plasma matrix, [^{125}I]-ACTH Ab, C-terminal anti-ACTH, coupled to sheep anti-rabbit coated tubes, X20 wash concentrate.) *Euro-Diagnostica B.V., Arnhem, The Netherlands.*

Oestradiol ELISA

(Containing 96 microtiter wells, coated with anti-oestradiol serum, estradiol conjugated to horseradish peroxidase, reference standard set, 0-2000pg/ml, substrate solution (TMB), stop solution and X40 wash solution.)

Steroid free human serum.

Immunodiasgnostic Systems, Tyne & Wear, UK.

Infinity Glucose Reagent

(Containing 2.1mM ATP, 2.5mM NAD, >1500U/L yeast hexokinase, >3200 U/l Glucose-6-phosphate dehydrogenase)

β-Hydroxybutyrate Assay kit

(Containing β-HBA reagent (4.6 mM NAD, oxamic acid and buffer, pH 7.6), 50 units/ml β-hydroxybutyrate dehydrogenase, pH7.6, 50mg/ml D-β-Hydroxybutyrate Calibrator)

10, 25 & 75mg/ml D-β-Hydroxybutyrate Calibrators

Sigma Diagnostics

Angiotensinogen and Renin Assays

Norit Charcoal	*Norit N.V., Amersfoort, The Netherlands*
Dextran T70	*Pharmacia AB, Uppsala, Sweden.*
Porcine Kidney Renin (1U; liberates 100µg of Angiotensin II from Angiotensinogen per hour at pH 6 and 37°C)	*Sigma*
RIA cups (No. 73.1055)	*Sarstedt, Beaumont Leys, Leicester, UK.*
Angiotensin I standard (1µg/ml) Anti-Angiotensin I Ab Renin Inhibitor Bilaterally nephrectomised rat plasma	*Generous gifts from Dr. C.J. Kenyon*

2.1.9 Materials for Animal Surgery

Alzet® Mini-Osmotic Pumps, Model 2002 (0.5µl/Hr for 14 days)	*Alza Corporation, Palo Alto, CA 94303, USA.*
All surgical instruments	*Fine Science Tools, Haverhill, Suffolk, UK.*

Catheters for Carotid Cannulation
(Non-sterile polythene tubing (o.d. 0.38mm, i.d. 1.09mm; ref. 800/110/120) inserted a distance of 0.2cm into the flared end of a translucent thin walled PVC tube (o.d. 0.63mm, i.d. 1.4mm;ref. 800/010/100/800). *Portex Ltd., Hythe, Kent, UK.*

Harvard Apparatus Vet Fluosorber Fluovac International Market Supply	*Shirley Aldred & Co. Ltd., Bradwell, Sheffield, UK.*
All syringes, hypodermic needles, venflons and suture material.	*Surgical Supplies Services Ltd., Cumbernauld, Glasgow, Scotland, UK.*

2.1.10 Equipment

TaqMan™ ABI Prism 7700 Sequence Detector™	*Applied Biosystems, UK.*
MacClassic II/ MacLab/4e (utilised for mesenteric perfusion experiments)	*ADInstruments Ltd., Chalgrove, Oxfordshire, UK.*
GeneQuant RNA/DNA Calculator	*Amersham Pharmacia Biotech.*
Berthold LB 2111 γ-counter	*Berthold Technology Ltd., St. Albans, Herftfordshire, UK.*
Thermacage Incubator (utilised to warm rats prior to tail-cuff plethsymography)	*Beta Medical and Scientific, Sale, UK.*

Optima™ TLX Ultracentrifuge (rpm >14000) Beckman J2-MC Centrifuge (utilised for maxi-preps and the preparation of competent cells)	*Beckman Instruments, High Wycombe, Buckinghamshire, UK.*
Electrophoresis Power Pac 300 Model 583 Gel Dryer	*Bio-Rad Laboratories Ltd.*
EL 312e Bio-Kinetics Microplate Reader	*Bio-Tek Instruments Inc., Winooski, Vermont, USA.*
Dage MTI CCD72S imaging camera	*Dage Inc., Michigan City, Indiana, USA.*
Elcomatic EM 720, pressure transducer (utilised in mesenteric perfusions)	*Elcomatic Ltd., UK.*
Eppendorf Mastercycler gradient (PCRs, RTs and RNA denaturation)	*Eppendorf AG, Hamburg, Germany.*
Whirlimixer	*FSA Laboratory Supplies, Fissons plc., UK.*
Phosphoimager screens	*Fuji Photo Film Company Ltd., Toyko, Japan.*
Peristaltic pump (utilised to perfuse mesenteric vasculature)	*Gilson Minipuls, Villiers, France.*

Labofuge 400R Centrifuge (utilised for 15 and 50ml volume Falcon tubes)	*Heraeus, Brentwood, Essex, UK.*
Ultra-Turrax T8 auto-homogeniser	*Ika, Labortechnik, Staufen, Germany.*
Northern light B95 Precision Illuminator	*Imaging Research Inc., St. Catherine's Ontario, Canada.*
IEC-Centra-8R centrifuge	*International Equipment Company, (division of Danon) USA.*
Lectromed Mutitrace 2 (chart recorder that was coupled to pressure transducer)	*Lectromed, Letchworth, UK.*
Leica Cryostat	*Leica Microsystems, UK.*
Phosphoimager FLA-2000	*Raytest Scientific Ltd.*
SensorNor 840, Physiological Pressure Transducer. (utilised to measure arterial pressure)	*SensoNor a.s., Horten, Norway.*
UV-160A UV-Visible recording Spectrophotometer	*Shimadzu Europa, Milton Keynes, Buckinhamshire, UK.*
Vacuum Pump	*Skatron Instruments, Dolasketta, Lier, Norway.*

Dri-block DB Series Photarc Dishwarmer 2 Sample Concentrator (utilised for extraction of steroids from rat serum)	*Techne UK Ltd., Newcastle,* *Staffordshire, UK.*
1450 Microbeta Plus Liquid Scintillation Counter (utilised for standard cpm counting)	*Wallac Oy.*

2.1.11 Software

Sequence Detector 1.6.3 software	*Applied Biosystems, UK.*
Dataquest™ A.R.T.™ 2.1	*Data Sciences International, MN, USA.*
MCID-M4 Image Analysis V.3.0 Rev 1.5	*Imaging Research Inc.*
MacLab Chart V 3.3.5	*ADInstruments Ltd.*
Fujifilm Fluorescent Image Analyser FLA-2000 V.1.0 Aida 2.0 Auto Image Data Analyser	*Raytest Scientific Ltd., Sheffield, UK.*
Stastistica v.5.0	*Statsoft, Tulsa, Oklahoma, USA.*
Mulitcalc Advanced v2.0	*Wallac Oy.*

2.1.12 Buffers and Solutions

Borate Buffer: 8.25g Boric Acid and 2.7g sodium hydroxide dissolved in 1l dH_2O and set to pH 7.4 with hydrochloric acid.

Caesium chloride/TE buffer: 100g CsCl dissolved in 100ml TE buffer.

Charcoal: 6.07g of TRIZMA base added to 0.5l distilled water, 5ml 5M HCL, 2g neomycin sulphate, 1.85g EDTA, 5g BSA, made up to 1l, pH 7.4 and stored in 100ml aliquots at -80°C. Prior to use; 0.062g Dextran T70 dissolved in 5ml of the above buffer. Final solution containing 0.6g Norit charcoal, 95ml buffer and 5ml Dextran T70 solution, kept at 5°C.

Deionised Formamide: 150ml formamide mixed with 15g mixed bed ion-exchange resin for >1hr, filtered twice and stored protected from light.

DEPC-treated water: Distilled water mixed with diethylpyrocarbonate (DEPC; 1 drop/ 100 ml), shaken and left for 1-24 hours prior to autoclaving.

1Kb DNA ladder: 20µg 1Kb ladder (Gibco, UK), in 200µl distilled water with 10% (v/ v) loading buffer.

GTE: 50mM glucose, 25mM tris, 10mM EDTA in dH_2O, adjusted to pH 8 with hydrochloric acid.

Loading buffers: *(a)* 40% sucrose w/ v, 0.25% bromophenol blue (w/ v) in dH_2O; (b) 0.35% w/v orange G, 40% glycerol in dH_2O.

LB Agar: LB broth plus 15g of agar/l added prior to autoclaving.

LB broth: 10g bactotryptone, 5g bacto yeast extract, 5g sodium chloride in 1l dH_2O, autoclaved immediately.

10X MOPS buffer: 42g MOPS, 16.6ml 3M sodium acetate and 20ml 0.5M EDTA dissolved in 1l dH_2O; adjusted to pH 7.

Phosphate buffered Saline (PBS): 0.1M phosphate buffer with 137mM NaCl, 2.7mM KCl in distilled water, pH 7.4, autoclaved before use.

Physiological Salt Solution (PSS; 'Krebs' bicarbonate solution): 123mM NaCl, 2.65mM KCl, 1.2mM KH_2PO_4, 1.29mM $CaCl_2$, 1.2mM $MgSO_4$, 25mM $NaHCO_3$ in distilled water, pH 7.4. Stored at 4°C and supplemented with 11.1mM glucose and 18μM ascorbic acid glucose immediately before use, continuously oxygenated by bubbling 95% O_2: 5% CO_2.

5M Potassium acetate: 245.6g potassium acetate dissolved in 300ml dH_2O, 57.5ml glacial acetic acid and 142.5 ml water (final volume 500ml).

10x Reverse Transcription buffer: 0.1M Tris-HCl, 0.5M KCl, 1% Triton X (ready mixed from Promega).

SDS solution: 0.2M sodium hydroxide, 1% w/v SDS.

Thermophilic DNA polymerase 10x reaction buffer: 500mM KCl, 100mM Tris-HCl and 1% Triton X (ready mixed from Promega).

5xTranscription optimised buffer: 200mM Tris-HCl, 50mM NaCl, 30mM $MgCl_2$, and 10mM spermidine (ready mixed from Promega).

10xTBE buffer: 0.9M TRIZMA base, 0.9M Boric acid, 20mM K_2-EDTA in distilled water.

TE buffer: 10mM tris, 1mM EDTA, adjusted to pH 8 with concentrated hydrochloric acid; autoclaved prior to use.

50mM Tris buffer: 12.14g of TRIZMA base added to 1l distilled water, 10ml 5M HCL, 3.5g neomycin sulphate, 1.75g Human serum albumin, made up to 2l, pH7.4, stored in 10ml aliquots at -80°C.

Ultra-pure water: dH_2O, UV-treated and autoclaved.

2.1.12 Drugs used in Mesenteric Perfusion Studies

Arginine Vasopressin acetate (AVP): 216.8mg dissolved in 20ml-distilled water (final concentration 10^{-4}). 1ml aliquots stored at -20°C. AVP was diluted to concentrations between 0.2 and 20nM in PSS prior to use.

Noradrenaline (NA): 95.79mg dissolved in 30ml-distilled water (final concentration 10^{-2}M). 1ml aliquots stored at -20°C. NA was diluted to concentrations between 0.2 and 20μM in PSS immediately prior to use.

2M Potassium Chloride (KCl): 14.91g dissolved in 100ml distilled water. Stored at 4°C. Prior to use, this solution was substituted for NaCl in the PSS to a final concentration of potassium between 25 and 125mM.

2.1.14 Drugs used in surgery

Nubain: One ml volume of strawberry jelly per average weight of rat was given, i.e. 0.5mg of Nubain/ml of jelly/250g rat. The jelly recipe was prepared as directed to the part where the jelly is dissolved in hot liquid. The volume of dissolvent used was reduced by the volume of Nubain to be added. When the mixture was <30°C, the Nubain was added, and once the jelly had set, it was placed on the cage floor.

2.2 Methods

2.3 Animals

All animal procedures were performed under the terms of the Animals (Scientific Procedures) Act 1986 and Project Licence number 60/2466. I was responsible for the maintenance of and prenatal injections given to the rats, with assistance from Mr. Keith Chalmers, Mr Donald Hay, Mr Willy Mungall and Ms Sharon Rossiter.

Animals were principally under my care, with assistance from the animal technicians of the Biomedical Research Facility throughout all experiments. Wistar rats were utilised in each experiment. All rats were maintained under controlled conditions of light (lights on 08.00 h – 20.00 h) and temperature (21-22°C), and allowed free access to standard chow and drinking water. Adult and post weaning rats were kept up to a maximum of 8 per cage and cleaned out weekly. Rat pups were kept with their mothers until postnatal day 21 and were not cleaned during this period.

"Bought in" rats were allowed to acclimatise to their new environment for a minimum of one week before mating. Single virgin females were housed with a male for the purposes of mating which was deemed successful when an expelled vaginal plug was observed. This was designated day zero of pregnancy. Males were used to impregnate a maximum of two females. Pregnant females were housed singly during gestation. Litters were weighed individually at birth and culled to eight, retaining either as many males as possible or in latter cohorts equal numbers of males and females. Pups were weaned at postnatal day 21 and separated according to sex for housing with littermates where possible. Offspring were weighed at this time and at regular intervals throughout postnatal life. In all experiments offspring were selected randomly from as many litters as possible, when this was impossible, a maximum of two animals from each litter were utilised per experiment.

2.3.1 Prenatal administration of dexamethasone

Prenatal administration of dexamethasone was preformed as previously described (Nyirenda *et al.* 1998). Briefly, dams were treated with daily morning subcutaneous injections of dexamethasone (100μg/kg/d) in 0.9% saline / 4% ethanol during the last week of gestation i.e. day 15 to day 21 inclusive. Control animals received equivalent injections of vehicle (1ml/kg, 0.9% saline / 4% ethanol) during the same time period.

Following successful pregnancy and birth, all "bought in" breeding pairs were sacrificed by cervical dislocation. Subject offspring were killed in a similar fashion or by decapitation. Relevant tissues were dissected, cleaned and weighed when necessary and quickly frozen on dry ice. Trunk blood was collected into a plastic tube containing ~0.1ml of 100mM EDTA via an EDTA-coated funnel, and quickly placed on ice. The tubes were then centrifuged at 2000g for 10 minutes, and the supernatant plasma transferred to Eppendorf tubes. Plasma and tissue were stored at -80°C.

2.3.2 Staging the oestrous cycle

To stage the oestrous cycle, 1ml of sterile saline was introduced to the rat's vagina and epithelial cells were aspirated and placed on a glass slide for microscopic inspection. The oestrous cycle was then staged according to the Journal of Animal Technicians Association, **12**.No.1. Staging was independently verified by at least one other researcher/animal technician.

2.3.3 Glucose tolerance tests

Rats were fasted from 17.00h to 09.00h, weighed and a basal blood sample (t = 0) taken by tail tip (i.e. removal of the distal 5-10mm of their tails). The rats were then administered 2g/kg glucose by gavage and returned to their cages. At 30 and 120

mins post glucose administration, additional blood samples were taken by 'tail-milking' (i.e. massaging the tail distally). All blood samples were collected into heparinised Eppendorf tubes, and treated as outlined above for the collection of plasma. Samples were frozen at -80°C. At the end of the glucose tolerance test and after bandaging their tails, the rats were allowed free access to food and water.

2.3.4 Pre- and post-operative care of animals

Prior to each surgical procedure, all animals were anaesthetised with Halothane and the appropriate surgical sites shaved and swabbed with ethanol. The rat was then placed on a warm plate, to maintain body temperature; additional heat was provided by the use of angle poise lamps. Immediately before the first incision and at regular intervals during the procedure, the depth of anaesthesia was determined by pinching the rat's foot tightly and observing any changes in respiration.

Postoperatively, all animals were placed in a cage on their own, under an infrared light. Each cage contained dishes of water, food and analgesia in jelly (nubain), thereby allowing the rat to self-medicate. The following day the animals were closely inspected for any signs of infection/discomfort and if healthy, were moved to an appropriate experimental cage.

2.4 Procedures for Blood Pressure Measurement

Both indirect and direct methods of blood pressure measurement were utilised. Blood pressure measurements were made in the first instance when the animals were three months old, to test for the programming phenotype, and then during subsequent experimental procedures.

2.4.1 Tail Cuff Occlusion Plethysmography

In general, the indirect blood pressure measurement techniques used for rats allow the investigator to measure systolic blood pressure over a long period of time in the same conscious animal without the need for surgical operation or anaesthesia. However, its accuracy is limited by any movement of the animal, the necessity for preheating the rat, and of course, stress. Such indirect systems require an appropriate cuff for occluding the arterial supply to the tail. The inflatable cuff, which is applied to the proximal end of the tail, is connected to a mercury manometer and the pulse wave generated is viewed on a computer screen. The apparatus used is as described by Evans, who found high correlation between the BP values obtained by plethysmography and arterial cannulation (Evans *et al.* 1994).

Before using the tail cuff, the animals were placed in a heating chamber at 37°C for ~3-10mins. This was to ensure release of the arterial sphincter at the base of the tail, thereby allowing arterial pulsations to be easily detected by the sensor. The animal was then wrapped in a towel to restrict movement and placed on a hot pad to maintain tail arterial vasodilation. An average of four deflation pressures were used to determine the animal's BP. If there was a difference of 10mmHg between the highest and lowest readings, the measurement was repeated. It should be noted that no degree of familiarisation was observed with this technique, and furthermore, no differences in BP values were noted between measurements at the beginning or end of the week.

2.4.2 Carotid Cannulation

Direct measurement of arterial pressure in the rat involves surgical cannulation of a major artery; in our case the left carotid artery was utilised, with the cannula later connected to a pressure transducer. This approach provides accurate measurements of both systolic and diastolic pressure (and therefore calculation of the mean arterial blood pressure). However, since these experiments are acutely performed, the blood pressure will be influenced to some degree by the surgery and anaesthesia. Therefore

in these experiments, the first measurements of BP were taken at least 30hrs following implantation of the cannula, when there were no remaining anaesthetic effects. Readings were taken over a period of 10mins, with the animal in a quiescent and unrestrained state.

The characteristics of an ideal catheter include: thromboresistance, ease of insertion, long-term tolerance, infection control and favourable mechanical and convenience factors. For these experiments, the catheters were made from polyethene material, as they have good flexibility, can be easily moulded and are rigid enough to be handled easily. Their main disadvantage is that they cannot be autoclaved for sterilisation. Catheters were prepared as follows: 2cm of non-sterile Portex® polythene tubing (o.d. 0.38mm, i.d. 1.09mm) was inserted a distance of 0.2cm into the flared end of a translucent thin walled Portex® PVC tube (o.d. 0.63mm, i.d. 1.4mm). The tubing was flushed and filled with sterile saline and clamped distally with forceps, ready for use.

The carotid artery lies medial to and below the jugular vein and was exposed by blunt dissection between the sternohyoid, syternomastoid and omohyoid muscles. Extreme care was taken to gently dissect the vagus nerve away from the artery (any damage to the nerve is readily visible post-operatively, as the rat displays signs of pstosis). In general the left carotid was employed as passage of the catheter along the right carotid may result in it emerging in the left ventricle, thereby causing arrhythmias. A catheter in the artery on the left side will pass into the aortic arch with no ensuing problems. An occluding ligature was placed as anteriorly as possible and a loose posterior ligature was positioned several millimetres away. A small bulldog clip was placed on the most distal end of the artery to stop the flow of blood. Using microscissors, a small incision was made in the artery and the catheter was fed through. The posterior ligature was lightly tightened round the artery and the catheter was pushed towards the heart while the clamp was released. The posterior ligature was then tied and secured around the artery and catheter. To exteriorise the catheter, a trocar of 18G was passed subcutaneously from the site of site of entry of the catheter to emerge in the dorsal nape of the neck. The end of the catheter was then

passed subcutaneously through the trocar needle. It was ensured that a loop of catheter was prepared near the neck incision site, to allow for normal body movements; without it, there is danger of the animal asphyxiating or pulling the tip of the catheter back or out of the vessel. After exiting, the catheter was then trimmed to ~25mm, stoppered with a pin fashioned from a suitably sized hypodermic needle and sutured into position. These sutures were further secured by the application of surgical glue, to prevent the animal from chewing or pulling at the exteriorised portion of the catheter. Finally, the various neck muscles were sutured back into their original positions and all skin incisions were either sutured or stapled.

To ensure and maintain catheter patency, it was flushed in the first instance with 0.2ml heparin (20 units heparin/ml) post-exteriorisation and then every day following BP measurement.

2.4.3 Radio-telemetry

Whilst the above techniques are commonly used to monitor blood pressure and heart rate in experimental models of hypertension, they are far from ideal. The use of tethers (to secure exteriorised vascular catheters) and both the warming and restraint required for 'non-invasive' tail cuff measurement, introduce a significant stress artefact, with well documented elevations of plasma cortisol and cathecholmaines. These problems may be overcome by using radio-telemetry, which measures BP (systolic, diastolic, and mean arterial blood pressure), heart rate and provides an estimate of activity in unrestrained conscious animals. It further affords the ability to collect data continuously over long periods of time, without the stress of human contact. Also, since rats are nocturnal animals, measuring their blood pressure at nighttime may prove more physiologically relevant and identify greater differences between the programmed and control animals.

The monitoring system consists of a surgically implanted transmitter (Data Sciences International PA11-C40), receiver and personal computer with accompanying

software (Dataquest Software) to decode the signal and measure the animals' activity, heart rate and systolic, diastolic and mean arterial BP.

Surgical implantation of the transmitter was kindly performed by Mrs G. Brooker. All animals were prepared for surgery and anaesthetised as described before. Following laporotomy, sterile saline soaked cotton wool was used to retract the gut in order to expose the abdominal aorta, which was separated gently from the vena cava. Two ties were placed as far proximally and distally as possible on the abdominal aorta in order to place tension on the vessel. A 21G needle was angulated at 45° and used to pierce the vessel wall and to introduce the probe. Once the tip of the probe was satisfactorily in place, the needle was withdrawn, the vessel sealed with a patch of cellulose (supplied with the transmitter) and the ties removed. The activity and position of the probe was tested by sweeping an active standard AM/FM Radio over the vessel; the implantation was considered successful if the probe disrupted the radio's signal. The transmitter's battery was then sutured firmly to the left abdominal wall, the intestines were repositioned and the surgical site closed with sutures and staples. Animals were cared for postoperatively as previously described.

No measurement was recorded until preliminary tests revealed that the heart rate and blood pressure had stabilised post-operatively. Animals were housed singly and each cage was placed over an individual receiver panel. Each cage and its associated receiver panel were housed in a specially designed steel rack, preventing any cross-talk between the radio-signals of neighbouring animals. The rack was stored in a quiet room within the animal facility, dedicated to telemetry measurement. Haemodynamic data was then continuously sampled every 30mins for periods of 10 seconds.

2.5 Mesenteric Perfusions

Isolation of the mesenteric vasculature was kindly performed by Ms. Sharon Rossiter. Pairs of rats (one control, one prenatally treated with Dex) were anaesthetised with sodium pentobarbitone and laparotomised, exposing the

intestines, aorta and subsequently the superior mesenteric artery. The aorta was ligated proximally to the superior mesenteric artery in order to minimise bleeding and the superior mesenteric artery was cannulated using a 18G Venflon, that was tied in place and perfused continuously with oxygenated PSS at a rate of $3ml.min^{-1}$, using a peristaltic pump.

The vasculature was isolated as previously described by McGregor in 1965 (McGregor 1965). Briefly, the mesentery was dissected and the gut removed from the duodenum to the end of the ileum using surgical scissors. The gut itself was then removed and the remaining isolated superior mesenteric arterial cascade was removed to a petri dish, which was kept at 37°C by a water bath. On alternate days, mesenteric preparations from each treatment group were attached to alternate transducers to exclude the possibility of any positional artefact. Tissues were maintained in buffer in covered Petri dishes while perfusions were carried out.

Perfusion pressure into the mesenteric vasculature was measured continuously through an Elcomatic EM 720 transducer (calibrated with a mercury sphygmomanometer) and decoded and recorded on a MacLab Chart V3.3.5. programme. Following a 15min equilibration period, a brief 'wake-up' bolus of 20nM NA was administered. In every experiment the expected minimal response of an 80mmHg rise in pressure was observed.

2.5.1 Vascular responses to NA, AVP & KCl

A pair of mesenteric vasculature preparations was simultaneously tested with increasing concentrations of agonists in the following order: NA (0.1, 0.2, 1, 2, 5, 10 and 20μM), AVP (0.5, 1, 2 5, 10, 20 nM) and KCl (25, 45, 65, 85, 105 and 125mM), all prepared in PSS. Vasculatures were perfused with increasing concentrations of agonist for three mins. Between tests, the vasculatures were infused with PSS alone, during which time the perfusion pressure returned to basal values. A recovery period of at least 30mins was allowed between each agonist.

2.6 Induction of stress by disturbance, weighing, and restraint procedure.

To assess responses to stress, offspring were subjected to a series of graded stressors from simple disturbance (a researcher entering their room), to being weighed, and placed in a Perspex cylinder for 15min. To evaluate the stress response to weighing, haemodynamic measurements were recorded prior to removal of the rat from its cage, and again immediately and at 5min post-weighing, when the animal was replaced in its home cage. For the restraint procedure, haemodynamic measurements were collected prior to the rat being placed in the cylinder, throughout the 15min restraint period, and at 15min post-restraint, when the animal was returned to its cage. A rest period of two days was allowed between experiments.

2.7 Haemodynamic responses to alterations in catecholaminergic mechanisms.

Haemodynamic responses were analysed 2 hr following the depletion of catecholamines by i.p. reserpine ($0.5mg.kg^{-1}$), and during a 15 min restraint test, performed 4 hr post-resperine administration. After a 1-week recovery period, haemodynamic responses were collected for 15 min following a low dose of i.p. d-amphetamine ($0.5mg.kg^{-1}$), aimed to cause systemic catecholamine release.

2.8 Plasma Assays

2.8.1 ACTH Immunoradiometric Assay

ACTH IMRA was performed using a commercially available kit (ACTH, coated tube system; Euro-Diagnostica, UK). All plasma samples were collected and stored as previously described, from the same animals used to obtain plasma for corticosterone measurement (see below).

Concentrations of ACTH in the range of 0-1250pg/ml were prepared in order to construct a standard curve. Standards (50µl) in duplicate and samples (50µl, neat plasma) in either singulate or duplicate were incubated in coated tubes (C-terminal anti-ACTH, coupled to sheep anti-rabbit) with 50µl ^{125}I ACTH antibody and left at room temperature overnight. Two tubes were prepared with 50µl ^{125}I ACTH antibody alone, in order to give total counts. The tubes were washed twice in 2mls of the supplied wash buffer before being thoroughly decanted and counted in a in a γ-counter. The concentration of ACTH in the samples was estimated by comparison with the standard curve. The inter- and intra-assay coefficients of variation were <10%.

2.8.2 Aldosterone radio-immunoassay

Aldosterone RIA was performed using a commercially available kit (Coat-A-Count Aldosterone, DPC, USA). A range of concentrations of aldosterone was prepared (0-3,300 pmol/l) to allow the construction of a standard curve. Standards and samples were incubated in duplicate aldosterone-specific antibody coated polypropylene tubes with ^{125}I Aldosterone (1 ml per sample) at room temperature for 18hrs. Two tubes were prepared with only ^{125}I Aldosterone to give total counts, and two with a mixture of ^{125}I Aldosterone and non-specific binding buffer to give non-specific binding. The tubes were decanted thoroughly before being counted in a γ-counter and the concentration of aldosterone in the samples estimated by comparison with the

standard curve. The inter- and intra-assay coefficients of variation were <15% and <10% respectively.

2.8.3 Angiotensinogen Radioimmunoassay

Plasma samples were diluted 1 in 400 in Tris buffer. A range of concentrations of Angiotensin 1 standards (0-400pg/10µl) was prepared to allow the construction of a standard curve. 10µl of standard together with 10µl of trapping agent (anti-Angiotensin 1) were incubated in duplicate in RIA cups at 0°C. 3µl of sample were also incubated in a similar fashion, with 5µl of a 1 in 30 dilution of Porcine Kidney Renin, and 10µl of the trapping agent. All standards and samples were centrifuged at 2000rpm in a Centra-8R centrifuge for 1min at 0°C before being transferred to a preheated 96 RIA cup holder, and shook for 1hr at 37°C. The 96 RIA cup holder was subsequently placed on ice, and 150µl of Tris, containing approximately 4-5000cpm ^{125}I and 10µl of renin inhibitor was added to each cup. Two cups were prepared with just 150µl of the Tris/^{125}I/renin inhibitor mix in order to calculate the total counts. A further 2 cups of sample were prepared as outlined above except for the addition of the Porcine Kidney Renin; this was to ensure that the samples were not generating Angiotensin I with endogenous renin under the above experimental conditions. All cups were incubated at 4°C overnight. 250µl of charcoal (continually stirred at 5°C, on a bed of ice and water) was added to each cup and centrifuged at 2500rpm in a Centra-8R centrifuge for 15min at 4°C. The supernatant was aspirated fully and the free charcoal pellet was counted in a γ-counter. The concentration of Angiotensinogen in the samples was estimated by inference from the Angiotensin I generated from comparison with the standard curve.

2.8.4 Corticosterone Radioimmunoassay

All animals were handled daily for a period of at least two weeks and all blood samples were obtained by tail nick (described above) between 07.00h and 08.30h and/or 19.00h and 20.30h.

Plasma samples were diluted 1 in 10 in borate buffer and denatured at 65°C for 30mins to dissociate corticosterone (B) from its carrier proteins. A range of concentrations of B were prepared (0-320nM) to allow construction of a standard curve. Samples and standards were incubated in duplicate with a mixture of [^{3}H]-B (10,000cpm per sample) and rabbit anti-cort Ab (1 in 10,000 dilution) in borate buffer in a total volume of 70µl for 1h. 50µl of anti-rabbit scintillation proximity assay beads were then added to each sample and the samples sealed and incubated overnight. The SPA beads bind to the primary antibody and if the primary antibody is bound to [^{3}H]-B, the SPA beads cause scintillation of the radioactive signal. As the concentration of unlabelled B increases, there is competition between binding of the labelled and unlabelled B to the primary antibody, and the radioactive signal decreases. Samples were counted on a Wallac Microbeta Plus liquid scintillation counter and the concentration of B in each sample was determined from a graph of cpm versus [cort], generated using the Multicalc programme. The inter- and intra-assay coefficients of variation were <10%, as reported by Dr. C.J. Kenyon, who developed the assay. There is extremely weak cross-reactivity with progesterone, deoxycorticosterone and cortisol (all < 8%) when compared to B (100%) (MacPhee *et al.* 1989).

2.8.5 Glucose Assay

Plasma samples were incubated in duplicate with glucose hexokinase reagent for not less than 5min. The reagent causes any glucose present in the sample to be phosphorylated and NADH to be generated, thereby resulting in an increase in absorbance at 340nM. The absorbances of the samples at 340nM were measured using a Shimadzu uv/ visible recording spectrophotometer with a reagent blank. If the plasma sample was haemolysed, a plasma blank (prepared by diluting the plasma sample with water) rather than a reagent blank was used to correct for the colour in the sample. The amount of glucose in the sample was calculated by comparison with a standard curve (100-600 mg/dl glucose). The inter- and intra-assay coefficients of variation were <2%.

2.8.6 Insulin ELISA

Insulin ELISA was performed using a commercially available kit (Ultra Sensitive Rat Insulin ELISA Kit, Crystal Chem Inc., Chicago, Illinois, USA). Plasma samples were diluted 1 in 2 in the supplied buffer before assay. A range of concentrations of insulin was prepared (0.1 to 12.8 ng/ml) to allow the construction of a standard curve. Standards and samples (5µl) were incubated in duplicate in the antibody-coated microplate with 95µl of samples dilutent at 4°C for 2hrs. Plates were washed with the supplied washing buffer before the addition of 100µl of the Anti-rat Insulin enzyme conjugate. Following 30min incubation at room temperature, the plates were washed again. 100µl of enzyme substrate solution was added to each well; the plates were covered in aluminium foil and left for 40mins at room temperature before the final addition of 100µl of enzyme reaction stopping solution. The absorbance of the plate was measured at 450nm (subtracting wave length 630nm) and the concentration of insulin in the samples estimated by comparison with the standard curve. The inter- and intra-assay coefficients of variation were both <10%.

2.8.7 Oestradiol Assay

Oestradiol assay was performed using a commercially available kit (Estradiol ELISA, IDS, Tyne & Wear, UK.).

Prior to initiating the assay, steroid extraction from rat plasma was performed. 250µl of plasma was mixed with 1ml of ethylacetate in Corning 12X15mm glass tubes, vortexed and centrifuged at 2000rpm in a Labofuge 400R centrifuge for 15mins at RTP. 800µL of the organic supernatant was transferred to a fresh glass tube, without disturbing the solid layer. All samples were placed on a hot pad and air-dried under a Sample Concentrator for 20mins. When they were completely dry, the samples were reconstituted in 75µl of steroid free human serum (zero standard).

25µl of standard and sample were dispensed into the appropriate wells, followed by 200µl of the enzyme conjugate (oestradiol conjugated to horseradish peroxidase).

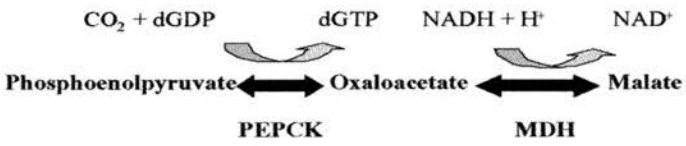
(400µl per well per wash). Plate contents were decanted and a 100µl per well of substrate solution (TMB) was added. The plate was incubated for 15mins at RTP before the enzymatic reaction was stopped by the addition of 50µl of stopping solution. The absorbance of the plate was measured at 450nm and the concentration of oestradiol in the samples estimated by comparison with the standard curve.

2.8.8 Phosphoenolpyruvate Carboxykinase (PEPCK) Assay

PEPCK is the rate limiting enzyme of gluconeogensis and catalyses the decarboxylation and phosphorylation of oxaloacetate to form phosphoenolpyruvate. In this reaction a nucleoside triphosphate is dephosphorylated to diphosphate. PEPCK can also catalyse the reverse reaction i.e. incorporation of CO_2 into phosphoenolpyruvate to yield oxaloacetate. The mammalian enzyme has a high specificity for nucleotides, accepting as substrates only inosine, guanosine and xanthosine nucleotides.

PEPCK activity can be measured by coupling the formation of oxalacetate in the backward reaction with NADH oxidation in the presence of excess malate dehydrogenase. However, in crude preparations, the relatively higher levels of tissue pyruvate kinase interfere in coupled assay system with malate dehydrogenase (IDP and GDP also being substrated for pyruvate kinase). dGDP has the unique property of being a good phosphate acceptor for PEPCK but is a poor substrate for pyruvate kinase, and, is therefore, used in the coupling reaction as shown in **Figure 2-1**.

Figure 2-1 Coupled reactions in PEPCK Assay



Phosphoenolpyruvate is first converted to oxaloacetate by PEPCK. Malate dehydrogenase (MDH) then reduces oxaoloacetate to malate using NADH as a co-factor. During this reaction an equimolar amount of NADH is oxidized to NAD^+. The consequent rate of decrease in absorbance at 340nm is directly proportional to the activity of PEPCK.

10% (w/v) fresh liver homogenates were prepared in ice-cold buffer (250mM sucrose and 5mM Hepes, pH 7.4). Homogenates were centrifuged for 10 min (4°C) at 10,000rpm to sediment intact cells, and the resulting supernatant was recentrifuged for 60min (4°C) at 50,000 rpm in a Beckman Optima TLX ultracentrifuge. The supernatant (cytosol) was collected and stored at -70°C and later used to assay PEPCK activity. Protein content of the samples was determined by the Bradford method.

PEPCK activity was measured at 30°C by coupling the formation of oxalocetate with NADH oxidation in the presence malate dehydrogenase, as described by Petrescu (Petrescu *et al.* 1979). The reaction mixture (1ml final volume) contained 50mM Hepes (pH 6.5), 50mM sodium bicarbonate, ImM $MnCl_2$, 0.25 mM NADH, 1mM phosphoenolpyruvate, 1.5U malate dehydrogenase and 100µL of liver cytosolic preparation. The reaction was initiated in a spectrophotometer, set to 'Kinetic' mode, with 0.15mM deoxy-GDP and the drop on absorbance at 340nm was followed for 5mins. A reaction mixture lacking bicarbonate was used as control. PEPCK activity was calculated from the rate of drop in absorbance at 340nm and the molar absorptivity of NADH.

together with 10μL of trapping agent (anti-Angiotensin I) were incubated in quadruplicate in RIA cups at 0°C. 2μl of neat plasma sample were also incubated in a similar fashion, with 8μL of substrate (bilaterally nephrectomised rat plasma), and 10μl of the trapping agent. All standards and samples were centrifuged at 2000rpm in a Centra-8R centrifuge for 1min at 0°C. Two of the four cups were incubated for 30mins at 0°C ('cold' standards and samples), whilst the remaining two cups were transferred to a preheated 96 RIA cup holder, and shook for 30mins at 37°C ('hot' standards and samples). All cups were subsequently placed on ice, and 150μl of Tris, containing approximately 4-5000cpm ^{125}I and 10μl of renin inhibitor was added to each cup. Four cups were prepared with just 150μl of the Tris/^{125}I/renin inhibitor mix in order to calculate the total counts. All cups were incubated at 4°C overnight. 250μl of charcoal (continually stirred at 5°C, on a bed of ice and water) was added to each cup and centrifuged at 2500rpm in a Centra-8R centrifuge for 15min at 4°C. The supernatant was aspirated fully and the free charcoal pellet was counted in a γ-counter. A standard curve was constructed using values obtained for the 'hot' standards and the concentration of Angiotensin I generated by both the 'hot' and 'cold' samples were made by comparison with this curve. To calculate renin activity in the samples, values for Angiotensin I generated by the 'cold' samples were subtracted from the values of Angiotensin I generated by the 'hot' standards.

2.9 Protein concentration by the Bradford method

The concentration of total protein in a solution was determined using the method of Bradford (1976). The assay is based upon the binding of Coomassie brilliant blue G-250 to basic and aromatic amino acid residues, and consequent colour change to the amount of protein of ≥ 3000kDa present. Samples were diluted with buffer to give solutions in the linear range of 0.05-0.5mg protein/ml, and BSA standards were prepared in the same buffer to 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml. Diluted protein assay dye reagent (1.96 ml) was added to 40μl of protein standard or appropriately diluted tissue homogenate in a borosillicate tube, vortexed to mix and left at room temperature for 15 min–1 hour to allow colour development. Absorbance of samples

at λ595nm was measured using a Shimadzu UV/ visible recording spectrophotometer and the concentration of protein in each sample was calculated from the mean absorbance, the dilution factor, and a graph of [protein standard] versus mean absorbance at λ595nm.

2.10 Northern Analysis of RNA

2.10.1 RNA Extraction

RNA extraction was perfomed using TRIzol® Reagent - a mono-phasic solution containing phenol and guanidine isothiocyanate. This reagent maintains RNA integrity whilst disrupting cells and dissolving cell components.

2.10.2 Homogenisation

To aid initial homogenisation, individual tissue/organs were wrapped in chilled aluminium foil whilst frozen. 1ml TRIzol® was added per 50-100mg tissue whilst frozen and samples were homogenised on ice using a mechanical homogeniser. Samples were centrifuged at 12,000g at 4°C for 10min to remove insoluble material and small amounts of unhomogenised tissue. The resulting supernatant was removed to sterile Eppendorf tubes.

2.10.3 Phase Separation

Following homogenisation, samples were allowed to equilibrate to RTP then left for 5 minutes to allow complete dissociation of the nucleoprotein complexes. 0.2ml Chloroform per ml TRIzol® used in original homogenisation was added to each sample. Samples were hand shaken vigorously for 15 seconds then incubated at RTP for 3 minutes. Samples were centrifuged at 12,000g at 4°C for 15min resulting in a lower red phenol-containing phase (containing proteins), an interphase (containing DNA and denatured proteins) and an upper aqueous phase containing RNA.

2.10.4 RNA Precipitation

The upper aqueous phase from each sample was transferred into a fresh eppendorf and the RNA was precipitated by addition of 500µl isopropanol (per ml TRIzol® in original homogenisation). Following addition of isopropanol, samples were incubated at RTP for 10min prior to centrifugation at 12,000g at 4°C for 10min. The RNA precipitate forms a visible gel-like pellet on the side of the tube.

2.10.5 RNA Wash

Following centrifugation the supernatant was removed and the RNA pellet was washed with 1ml 75% ethanol (per ml TRIzol® in original homogenisation). Pellets were vortexed and centrifuged at 9000g at 4°C for 5min.

2.10.6 RNA Resuspension

Following the RNA wash, the ethanol was removed and the pellets were briefly air-dried for 5min, being careful not to completely dry out the RNA, which greatly reduces solubility. RNA pellets were dissolved in 10-50µl DEPC-treated water (depending on pellet size) followed by incubation at 60°C for 10min. RNA was stored at -80°C until required.

2.10.7 RNA Quantification

Before use, RNA was quantified using a GeneQuant RNA/ DNA Calculator. RNA was diluted 1:25-1:100 in DEPC-treated water and the optical density at λ260nm and λ280nm was determined to assess concentration and purity. Only RNA with a Δ260/Δ280 of between 1.5 and 1.8 was used.

2.10.8 RNA electrophoresis and capillary transfer

Total RNA was separated by electrophoresis on a 1.2% agarose formaldehyde denaturing gel. A 100ml gel was prepared by melting 1.2g of agarose in 88ml DEPC-treated water, adding 40% formaldehyde (2ml) and 10X MOPS buffer (10ml), and pouring into a gel mould with appropriately sized combs in place. The gel was allowed to set before being soaked in 1X MOPS buffer in a gel tank for 30mins. RNA was prepared for electrophoresis by aliquoting 20µg of RNA, adding DEPC-treated water to bring the total volume to 10µl, then adding deionised formamide (10µl), 40% formaldehyde (2.5µl) and 10X MOPS buffer (2.5µl) to give a total volume of 25µl. The sample was mixed and the RNA was denatured by incubation at 65°C for 15mins. Ethidium bromide was added to the loading buffer (1µl per 50µl loading buffer) and 2µl of this mix was added to each sample of the denatured RNA. The RNA was loaded into the wells on the gel and electrophoresed at 100V for 2-4hrs, until the front band of the loading buffer was ¾ of the way down the gel. The gel was photographed under UV light (λ = 254nm) with as little exposure as possible

to minimise RNA damage. The presence of intact 28S, 18S and 5S Ribosomal RNA bands indicated that the preparation was undegraded. The gel was soaked in 20X SSC buffer for 15min before blotting onto a nylon membrane. A wick of Whatman 3MM filter paper was placed over an upturned gel mould in a plastic tray containing 20X SSC buffer and the gel placed on top. A piece of nylon membrane cut to the same size was smoothed on top of the gel and this was covered with 3 layers of 3MM filter paper and approximately 10cm of paper towels. A glass plate and light weight was placed on the top to secure the apparatus, and a builder's level was utilised to ensure "evenness" of the tower. Capillary transfer was allowed to take place overnight at room temperature. The following day, the membrane was washed in 20X SSC to remove any gel and the efficiency of transfer checked by photographing both the gel and the membrane under UV light (λ = 254nm). The membrane was dried between two sheets of 3MM filter paper at 80°C for 2hrs, and the RNA cross-linked under UV light.

2.10.9 Hybridisation to ^{32}P-labelled cDNA

The membrane was soaked in 20X SSC buffer for 5mins before being placed in a Hybaid hybridisation bottle containing 3mL 20% SDS and 6ml phosphate buffer that had been pre-heated to 55°C. Denatured Herring testes DNA (10mg/ml; 100µl) was then added to this. The membrane was prehybridised in a Hybaid hybridisation oven at 55°C for a minimum of 2hrs. The ^{32}P-labelled cDNA probe (prepared as described in section 2.11) was added to the hybridisation buffer and the membrane was hybridised with the probe overnight at 55°C. The probe was then disposed of and the membrane was rinsed with wash buffer one. This was followed by a further two washes in the same wash buffer at room temperature for 20 mins and one wash in wash buffer two for 30mins at 55°C. The level of radioactive signal remaining on the membrane was checked and if this was too high the final wash was repeated as necessary. The membrane was wrapped in parafilm and exposed to a Fujifilm imaging screen for a length of time between 5mins-24hrs and the level of hybridised probe quantified using a Fuji FLA2000 fluorescent image analyser. The membranes were then rehybridised with U1 cDNA in the same way to control for RNA loading

and transfer. The level of expression of the RNA of interest was then expressed as a ratio of the signal of the RNA of interest to the signal for U1. Membranes were left for a minimum of one month before reprobing to allow decay of the original signal and all mRNA species probed were well separated on the membrane. If results between groups of animals were to be compared all the samples were hybridised in the same hybridisation bottle with the same probe.

2.11 Preparation of ^{32}P-labelled cDNA probes

2.11.1 Preparation of cDNA templates by PCR

cDNA templates were prepared by PCR for various genes of interest in liver, kidney, fat and hypothalamus, to be used subsequently for the synthesis of DNA probes for use in Northern blots. Forward and reverse oligonucleotide primers were designed to target rat mRNA sequences of interest, based upon published sequences made available through the BLAST database (National Centre for Biotechnology Information, National Library of Medicine, Bethesda, MD, USA.).

2.11.2 Reverse Transcriptase Reaction

First strand cDNA synthesis was performed using Promega's Reverse Transcription System. 3μg of total RNA was reverse transcribed in a reaction mixture containing 25mM $MgCl_2$, 10X reverse transcription buffer, 10mM each of dATP, dCTP, dGTP and dTTP, 20U RNasin, 0.5μg Oligo$(dT)_{15}$ primers and 15U AMV-reverse transcriptase made up to 50μl in DEPC-treated water. Samples were incubated at 42°C for 50min followed by 5min at 99°C then 5min on ice to inactivate enzymes and prevent binding to DNA.

Negative control reactions for each RNA sample were performed in parallel (made up as above but in the absence of AMV-reverse transcriptase) in order to determine

genomic DNA contamination. A further negative control reaction containing water instead of RNA was performed to determine RNA contamination of the Reverse Transcriptase System reagents.

Plasmid DNA (10μg) was digested with the appropriate restriction enzyme (10 units) in 1 X restriction enzyme buffer in a total volume of 100μL for 2hrs at 37°C (see table). Digestion of the DNA was confirmed by electrophoresis of 3μL of the digest through a 1% agarose gel (prepared by dissolving 1% agarose in 1 TBE buffer and adding 1μl ethidium bromide (10mg/ml)). The digest was compared with uncut plasmid and a 1kB DNA ladder containing fragments ranging from 75-12kb under UV light at λ = 254nm. If the plasmid was sufficiently digested the remaining digest was electrophoresed in a large single well of a 1% low melting point agarose gel (made as above but using low melting point agarose).

The DNA fragment was visualised under UV light (λ = 254nm), excised from the gel using a scalpel, purified from the gel using Hybaid's Recovery DNA Purification kit and resuspended in 50μl DNAase free/ RNAase water. Recovery of the DNA fragment was assessed by electrophoresis of 1μl of the DNA solution through a 1% agarose gel as described below.

2.11.3 PCR Reactions

5μl of cDNA template was used in each PCR reaction containing 2.5μl of 10X Thermophilic DNA polymerase Reaction Buffer containing 25mM $MgCl_2$ and made up to a final volume of 25μl with DEPC-treated water. A second mixture containing 2.5μl of 10X Thermophilic DNA polymerase Reaction Buffer containing 25mM $MgCl_2$, 2.5mM each of dATP, dCTP, dGTP and dTTP, 15pmol upstream primer, and 15pmol downstream primer was made up to 25μl in DEPC-treated water. 0.25μl of *Taq* polymerase was added to each reaction tube just prior to starting the required PCR programme. A negative control reaction containing DEPC-treated water rather than cDNA was performed in parallel to determine contamination of PCR reagents.

PCRs were performed on an Eppendorf Mastercycler Gradient with a heated lid (set to 110°C). Samples were heated to 95°C for 3min for initial denaturation, placed directly on ice for 1min before the addition of the second mixture. All samples then underwent 35 cycles of PCR amplification (denaturation at 94°C for 1min, primer annealing at primer-specific temperature for 1min and elongation at 72°C for 2min). Upon completion of the PCR programme, samples were incubated at 72°C for a further 10min to ensure elongation of products to full length and chilled to 4°C prior to gel electrophoresis. Specific primer sequences and PCR conditions are detailed in **Table 2-1**.

2.11.4 Gel Electrophoresis

RT-PCR products were analysed by electrophoresis on a 1.0% agarose gel. Gels were prepared by melting 1% (w/v) agarose in 0.5X TBE and adding 1µl/ 100ml of ethidium bromide. After pouring into a gel mould with appropriately sized combs in place, gels were allowed to set at room temperature then placed in 0.5X TBE in a gel tank.

10µl 1Kb DNA ladder containing fragments ranging from 75-12,000 bases were loaded into the first well on each gel to allow determination of product size. 2-5µl of each RT-PCR product was mixed with 2.5µl loading buffer and loaded into individual wells of the gel. Gels were electrophoresed at 100V for 30-45min until the loading buffer band was approximately ¾ of the way down the gel and were then photographed under UV light at λ260nm.

Table 2-1 **PCR primer sequences**

	Sequence (5'-3')	cDNA Position	Accession number	Amplicon length
Mouse Angiotensinogen			NM007428	506
Forward primer	tgtacaagatgctgaatgagg	323-343		
Reverse primer	gtagggtgctggtctgtactg	783-802		
Mouse GAPDH			M32599	1001
Forward primer	gtcggtgtgaacggatttggccgt	51-79		
Reverse primer	catggcctacatggcctccaagg	1021-1043		

(Annealing temperature for all sequences = 56°C; 35 cycles)

Table 2-2 **Real-time PCR and Probe Sequences**

	Sequence (5'-3')	cDNA Position	Accession number	Amplicon length
Angiotensinogen			NM_134432	94
Forward primer	ttcacttccaagggaagatgaga	818-840		
Reverse primer	agcatgggcacagacactga	892-911		
Probe	tgctgttgtccacccagaactcatgg	865-889		
Cyclophilin			M195333	99
Forward primer	cccaccgtgttcttcgaca	52-71		
Reverse primer	aaagttttctgctgtctttggaact	126-151		
Probe	caagggctcgccatcagccgt	73-93		

(Annealing temperature for all sequences = 59°C, and probes = 69°C; 40 cycles)

2.11.5 Ligation of cDNA into vector

Purified PCR products were ligated into pGem-T Easy plasmid utilising –A/-T overhangs generated by the Taq polymerase. The volume of purified PCR product containing an amount of DNA equal to three times the molar quantitiy of vector per µl in the kit was calculated in each case. This volume was mixed with 5µl of X2 Ligase buffer, 1µl linear vector and 1µl of T4 DNA ligase and made up to 10µl with ultra-pure water. Positive and negative control ligations were set up, containing 2µl of control DNA insert in place of PCR product, or no DNA respectively. The mixtures were incubated at 4°C overnight.

2.11.6 Cloning of plasmid DNA

Bacterial transformation: *Eschericia coli* HB101 cells were grown in 100ml of LB broth at 37°C in a shaking incubator until they reached mid-log phase (A_{600} = 0.3-0.6). They were then centrifuged at 3000g for 5 mins at 4°C, the pellet re-suspended in cold calcium chloride (0.1M; 20ml) and left on ice between 10mins and 2hrs. The centrifugation step was repeated to re-pellet the cells and the cells were re-suspended in cold calcium chloride (0.1ml; 2ml). The competent cells were stored on ice in the fridge for up to 3 days before transformation.

Competent cells (100µl) were mixed with plasmid DNA (50ng) and left on ice in the fridge for 20mins. The cells were heat shocked at 40°C for one minute and replaced on ice. The heat shock and the calcium chloride lead to the incorporation of the plasmid DNA into the cells. The cells were spread onto LB agar plates containing 100µg/ml ampicillin, and the plates incubated overnight at 37°C. Only cells that have incorporated the plasmid DNA will grow on the ampicillin plates, as HB101 cells do not have inherent ampicillin resistance.

2.11.7 Plasmid DNA preparation

A single transformed bacterial colony was selected from an agar plate and incubated overnight at 37°C in 3mL LB containing 100μg/ml ampicillin. This was then added to 500ml LB containing 100μg/ml ampicillin and incubated overnight at 37°C. The culture was centrifuged at 6000 rpm for 5min at 4°C in a Beckman J14 centrifuge, and the supernatant discarded. The cell pellet was resuspended in 12ml cold GTE buffer, 24ml of cold alkaline SDS was added and the mixture left on ice for 10min. Cold potassium acetate (16ml) was added and the mixture left on ice for a further 10 min before being centrifuged at 6000 rpm for 5min at 4°C in a Beckman J14 centrifuge. The mixture was filtered through sterile gauze to remove the precipitate, isopropanol (32ml) was added to the filtrate and the mixture was left at room temperature for 30mins to precipitate the DNA. The DNA was pelleted by centrifugation at 10,000rpm for 3mins at 4°C in a Beckman J20 centrifuge, and the supernatant discarded. The DNA pellet was resuspended in 2.2ml TE buffer, 2.9g CsCl added and dissolved 100μl ethidium bromide (10mg/ml) added. The mixture was transferred to Beckman Quickseal ultracentrifuge tubes and topped up with CsCl/TE solution and centrifuged at 70,000rpm for 20hrs at 20°C in a Beckman Optima TLX ultracentrifuge. The DNA was separated into bands that could be visualised by the pink colour of the ethidium bromide. These DNA bands were removed by a 21G needle and syringe, transferred to fresh ultracentrifuge tubes, topped up with CsCl/TE solution and centrifuged at 100,000 rpm for 4hrs at 20°C in a Beckman Optima TLX ultracentrifuge. The DNA bands were collected as before and the ethidium bromide was removed by extracting repeatedly with water-saturated butanol until the pink colour disappeared. The DNA was precipitated with ethanol (2 volumes) for 10mins at room temperature and pelleted by centrifugation for 5mins at 14, 000g. The supernatant was discarded and the DNA pellet washed with 70% ethanol then resuspended in 1ml TE buffer. The concentration and purity of the DNA was assessed spectrophotometrically using the ratio of absorbances at λ = 260 and 280nm.

2.11.8 Restriction enzyme digestion of the plasmid, and purification of the fragment

Restriction enzyme digestion was used for the linearisation of insert-containing vector in advance of probe synthesis, the excision of inserts to verify their size and to determine the orientation of inserts by asymmetric digestion.

GR insert was excised from its pGEM-3 vector (generously donated by Dr. Karen Chapman) using Ava 1.

U1 insert was excised from its pBluescript KS+ vector (generously donated by Dr. Matt Sharp) using EcoR 1 and Hind 111.

In general terms, 1-2µl of enzyme was mixed with one-tenth the total volume of the supplied buffer and 1-5µg of DNA, ± BSA, and made up to between 20 and 60µl total volume with DEPC-treated water. This was incubated for 90 to 180 minutes at 37°C, and an aliquot electrophoresed on a 1% agarose gel to verify linearisation, orientation and to examine the length of products with reference to a DNA ladder.

2.11.9 Sequencing of cDNA

Was performed by DNAShef, at the Dept. of Haematology, Royal Infirmary, Edinburgh.

2.2.10 ^{32}P-labelling of cDNA

Roche's random primed DNA labelling kit was used to label the DNA fragments. Approximately 25ng of DNA fragment was aliquoted into an Eppendorf, made up to 10µl with DEPC-treated water and denatured at 100°C for 10mins. The Eppendorf was cooled on ice and briefly centrifuged. Hexanucleotide primer mix (2µl), dATP, dTTP, and dGTP (1µl of each), [α^{32}P]-dCTP (4µl) and Klenow (1µl) were added to give a total reaction volume of 20µl and the reaction incubated at 37°C for 1hr. Unincorporated radioactivity was removed by passing the mixture through a NICK column. The NICK column was prepared by washing with 3ml TE before application of the mixture. The column was washed with 400µl TE, the elutant discarded and the labelled DNA eluted from the column with a further 400µl TE. The activity of the probe was assessed by mixing 1µl of probe with 1ml of Cocktail T scintillant and counting in a β-counter. The probe was used if the specific activity was greater than 10,000cpm/µl. The DNA probe was denatured before use by heating to 100°C for 5min.

2.12 Real Time PCR

2.12.1 TaqMan™ probe and primer design

TaqMan™ probes and forward and reverse primers were designed using Primer Express™ software. Four basic rules were used as guidelines when designing and choosing the optimal sequences i.e.

1. Primers should be ≥ 18 base pairs long.
2. There should be no more than 3 G or C base pairs within the terminal 5 base pairs of the 3' ends of each primer.
3. T_m of the probe must be ≥ 10°C than the highest primer T_m.

4. There should be ideally more C than G residues in both the primers and probe.

All primer and probe sets were designed to run across different exons. BLAST searches of the probe and primer sequences revealed no significant homology to sequences other than the specific gene of interest. In any case, TaqMan™ RT-PCR will not give any measurable signal without all 3 of the probe/primers working for any one target. Prior to beginning the assay, the quality of the primers was assessed by amplifying the genes of interest by standard RT-PCR. Specific primer and probe sequences are outlined in **Table 2-2**.

2.12.2 TaqMan™ Real-Time RT-PCR Assay

The TaqMan™ assay requires both forward and reverse primers as well as a fluorogenic probe that anneals between the forward and reverse sites. The probe has a fluorescent reporter dye at the 5' end and a quencher dye at the 3' end. During PCR cycling the probe is cleaved by the 5'-3' nuclease activity of Taq polymerase, the reporter and quencher dyes become separated resulting in an increase in fluorescence. The ABI 7700 measures the accumulation of PCR product by continuously monitoring the increase in fluorescence during cycling. Cycle threshold (C_T) is set to a level above the baseline fluorescence and once chosen, sets the point at which the sample PCR amplification plot crosses threshold. The C_T value in turn is predictive of the quantity of input cDNA. The more of the target gene present in the sample the earlier it will be detected in the PCR cycles and the lower the C_T. C_T detection is converted into number of gene copies in the starting material and a standard curve is constructed using known amounts of rat genomic DNA. Test gene mRNA values are extrapolated from the standard curve and expressed in arbitrary units. The assay has been described in full elsewhere (Gibson *et al.* 1996; Heid *et al.*1996; Lie *et al.* 1998).

For the assay, 500ng of sample RNA was prepared and reversed transcribed as previously described. 5µl of cDNA from each sample was collected and used to form stock cDNA, from which a standard curve was created. All sample cDNAs were diluted 1:4 with nuclease-free water.

One TaqMan™ cDNA plate was used per test gene. For each gene of interest a primer/probe mix was created, consisting of (for 100 reactions): 30µl of 25µM forward primer, 30µl of 25µM reverse primer, 100µl of 5µM probe and 540µl of nuclease-free water. 550µl of this mix was added to 550µl of a cyclophilin primer/probe mix (internal control) and 1250µl TaqMan™ PCR core reagent, to form a master mix. Each sample was assayed in triplicate; 67.5µl of the master mix was added to 7.5µl of sample cDNA, and 24µl of this final mixture was added to three wells of the cDNA plate. In order to generate a standard curve; serial 1:10 dilutions of the stock cDNA were added to the master mix and added to the plate in a similar manner. Three no-template negative controls were constructed by substitution of nuclease-free water for cDNA. Four negative RT controls were further added to the cDNA plate. Three wells contained a 1 in 4 dilution of the stock cDNA and acted as quality control. Plates were analysed on a TaqMan™ ABI Prism 7700 Sequence Detector™. Cycling parameters: 50°C for 2mins, 95° for 10mins, 40 cycles of 95°C for 15s, 60°C for 1min. Data acquisition was processed with Sequence Detector 1.6.3 software. All controls and a selection of samples, chosen at random were electrophoresed on a 1% Agarose gel to verify specific amplification and to examine the length of products with reference to a DNA ladder.

2.12 ^{35}S In Situ Hybridisation

Please note: The hepatic GR *in situ* hybridisation was kindly performed by Mrs. Karen Anderson and evaluated by Dr. Megan C. Holmes.

In situ hybridisation allows the visualisation of the exact cellular and/ or structural location of specific mRNAs (indicating transcription of the corresponding gene) by hybridisation of a ^{35}S-labelled 'antisense' RNA probe to the mRNA of interest. ^{35}S-

UTP labelled RNA 'sense' probes of similar length, nucleotide content and specific activity were included in the experiment in order to assess the specificity of the hybridisation reaction.

Only RNase free, sterile solutions and equipment were used for *in situ* hybridisation experiments in order to prevent degradation of target mRNA by exogenous RNases.

2.13.1 Slide Preparation

Prior to use, glass microscope slides were coated in 3-aminopropyltriethoxysilane in order to prevent section dehiscence. Slides were racked and washed in the following series of solutions; 0.2M HCl for 3min, DEPC-treated water for 3min, 2% 3-aminopropyltriethoxysilane in acetone (filtered through $NaSO_4$) for 10s, acetone for 3min (twice), and finally DEPC-treated water for 3min. Slides were air-dried for 30-60min before baking at 50°C for 4-16 hours. Dried slides were wrapped in aluminium foil and stored for up to 3 months.

2.13.2 Tissue section preparation

Liver from female rats aged 6mo were frozen immediately on powdered dry ice and stored at -80°C until required.

Frozen tissue sections were cut using a Leica cryostat. Tissues frozen at -80°C were placed in the cryostat chamber at -20°C and allowed to equilibrate for approximately 30min. Following equilibration, tissues were embedded in Cryo-m-bed embedding compound and positioned in the correct orientation for sectioning. 15μm thick sections of brain were transferred to aminopropyltriethoxysilane-coated slides, and stored at -80°C until required.

2.13.3 Synthesis of ^{35}S-UTP Labelled Ribo-Probes

See Table 2-3 for specific characteristics of probe generation.

Table 2-3 Characteristics of Probe Generation for *In Situ* Hybridisation

Probe	RNA polymerase	Temp (°C)
Rat GR *as*	T7	37
Rat GR *s*	SP6	40

s = sense; *as* = antisense; nt = nucleotide.

0.5-1μg of linearised plasmid (described in section 2.11.8) was transcribed by incubation at the appropriate temperature for 60-90min with ATP, CTP and GTP (10mM each), ^{35}S-UTP (s.a. 800Ci/ mmol), 10mM dithiothreitol (DTT), 0.5μl RNase inhibitor, and 1μl appropriate polymerase in a total volume of 10μl 1X transcription optimised buffer. Following incubation, 1μl DNase 1 (RNase free) was added and reactions incubated at 37°C for a further 15min to degrade the DNA template, after which probes were placed on ice for 1-5min and purified using NICK columns, to remove unincorporated radioactivity. The column was prepared by washing through with 3ml TE buffer. The probe mixture was then applied to the column. The column was washed with 400μl TE buffer and the initial elutant discarded. Labelled probe was eluted in an additional 400μl TE buffer.

For each probe, the total activity was estimated by counting 1μl of probe in 1ml PicoFluor 40 scintillant fluid in duplicate in a β-counter (minimum activity required $2x10^5$ cpm/ μl). Probes were stored at -20°C until required, for a maximum of 7 days.

2.13.4 Fixation Protocol

Slides were removed from the -80°C freezer and kept on dry ice until the start of the fixation procedure. Slides were fixed in ice cold 4% paraformaldehyde in 0.1M phosphate buffer for 10min, rinsed twice in 1x PBS for 5min, acetylated in 0.1M triethanolamine with 0.25% acetic anhydride for 10min to prevent loss of signal and rinsed in 1x PBS for 3min. Following dehydration through a series of ethanol solutions (70, 80 and 95% ethanol in DEPC-treated water) slides were air dried for 30min.

2.13.5 Pre-hybridisation & Hybridisation Steps

Following fixation, slides were pre-hybridised with 200µl/ slide of 2x pre-hybridisation buffer diluted 1:1 with deionised formamide, at 50°C for 2h. Dampening two layers of Whatmans No.3 chromatography paper with box buffer humidified the slide boxes, hence preventing tissue sections from drying out.

Sense and antisense probes were thawed and added to 2x hybridisation buffer diluted 1:1 in deionised formamide to give a final probe concentration of $20x10^{-6}$ cpm/ml. Probes were denatured at 90°C for 10min and placed on ice before addition of 10mM DTT. Pre-hybridisation buffer was drained from slides and 200µl appropriate probe was applied to slides. Slides were hybridised in sealed, humidified boxes at 50°C for an optimum of 16 hours.

2.13.6 RNase Treatment & Washes

Following hybridisation, slides were washed three times in 2x SSC for 5min and carefully wiped dry around the sections with lens tissue. 200µl of RNase A (30mg/

ml in RNase buffer) were applied to each slide and slides were incubated at 37°C for 1 hour in humidified boxes (1 layer of Whatman No.3 chromatography paper dampened with RNase buffer) to remove unhybridised probe.

Following RNase treatment, slides were washed in 2x SSC at room temperature for 30min, then twice in 0.1x SSC at 60°C for 60min. After washes, slides were dehydrated through a series of ethanol solutions containing 0.3M ammonium acetate (2min in each of 50, 70 and 90% ethanol) and air-dried.

2.13.7 Visualisation of Hybridisation

Slides were exposed to Kodak Biomax-MR film for 2-7 days. For hepatic sections, slides were individually dipped in NTB-2 photographic emulsion (diluted 1:1 with DEPC-treated water at 42°C) and exposed in light-tight boxes for 5 days to 12 weeks at 4°C. Slides were developed in D19 solution (diluted 1:1 with water) at 15°C and fixed in Amfix solution (diluted 1:5 with water at 15°C). The hybridisation signal was quantified by counting silver grains in the liver under bright field illumination using a computer driven image analysis system (Carl Zeiss, Welwyn Garden City, UK). The analysis was carried out blind to prenatal treatment. For each animal, 20 hepatocytes were assessed. Results were calculated as mean number of grains per hepatocyte after background counted over non-specific areas was subtracted.

2.14 Statistics

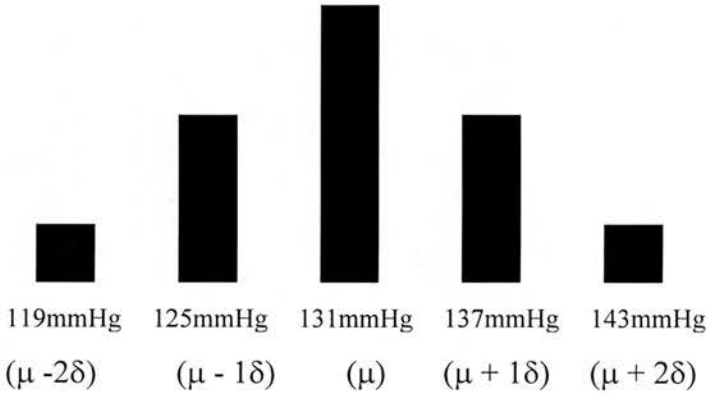
All values are expressed as mean ± standard error of the mean. Results were taken to be significant with $p \leq 0.05$. All analysis was performed using the Statistica programme.

The difference between the mean values of measurements made on two comparable treatment groups was tested by Student's t-test, and between multiple groups by One-Way ANOVA. When multiple separate treatment types were involved, Two or Three-Way ANOVA was performed, with *post-hoc* comparison of means testing using the LSD test or planned comparison. If measures were repeated, this was also taken into consideration.

As revealed by Figure 2-2, the systolic blood pressures obtained for both prenatally DEX and vehicle treated animals at 3 months of age, illustrates that their BP follows a Normal (or Gaussian) distribution.

Figure 2-2 Graph of Normal distribution for systolic blood pressure

Data taken from Figure 3-2 (page 89); mean (μ): 131 mmHg, Standard Deviation (δ): 5.7mmHg, N = 12, with 6 from each prenatal treatment group.



Determination of sample size (n) can be calculated from:

$$n = (z)^2(\delta)^2/ (d)^2$$

where: z = confidence coefficient

δ = standard deviation

d = interval

It is fair to assume an interval of about 8 units wide, that is, the estimate should be within about 4 units of the true value in either direction. We also assume a confidence coefficient of 0.95, and from Figure 2-2, we know that the standard deviation is 5.7mmHg. Therefore, z = 1.96, δ = 5.6, and d = 4.

On making the proper substitutions, the value of n is found to be:

$$n = (1.96)^2(5.7)^2/ (4)^2$$
$$= 7.53$$

Therefore, a sample size of 8 is justified for our experiments.

Note: A full derivation of the above equation, and the table of Normal Curve Areas are available in 'Biostatistics: A foundation for analysis in the health sciences' by Wayne W. Daniel, Pages 97-88, 143-145 and 491, 3rd edition, published by John Wiley & Sons.

Perinatal environmental stress profoundly affects glucocorticoid programming experiments

3.1 Introduction

As research institutions expand, and respond to ever-changing building and animal welfare regulations, they are required to undergo either new construction and / or renovation. However, such structural changes are associated with a plethora of nuisances, such as noise, vibration, and alterations in temperature and air-flow; and each disturbance is capable of powerfully stimulating the HPA axis (Dallman *et al.* 1999). The auditory system is permanently open - even during sleep. Its rapid and overshooting excitations caused by noise signals are subcortically connected via the amygdala to the HPA-axis, resulting in CRH and ACTH release (Spreng 2000a). Animal experiments show noise-induced changes in the sensitivity of GR by increase of heat-shock proteins (Spreng 2000a), and ultrastructural changes of the heart (Lenzi *et al.* 2003) and adrenal gland (Soldani *et al.* 1999). Increased cortisol levels have been found in humans when exposed to aircraft (Haines *et al.* 2001) or road traffic noise (Ising & Ising 2002), even during sleep (de Jong 1993), implying these effects are mainly without mental control. Of course, increased glucocorticoid and sympathetic neural secretion is a perfect short term stress response, co-ordinating appropriate metabolic and vascular changes, and thereby assisting the individual to negotiate the stressor. However, over prolonged time periods, such persistent noise-induced stress responses can be gravely damaging to health. The adverse effects of long term increases in cortisol (human) / corticosterone (rodent) are manifold: immunosupression (e.g. eospinopenia), insulin resistance (e.g. diabetes), cardiovascular disease (e.g. hypertension), catabolism (e.g. osteoporosis) and intestinal problems (e.g. stress-ulcer) (Spreng 2000b; Gamallo *et al.* 1992; Tomei *et al.* 1991; Sobrian *et al.* 1997; Kay *et al.* 1998). Worse still, may be the widespread extrahypothalamic effects of CRH/ and/ or ACTH which have the potential to influence nearly all regulatory systems, causing for example, stress-dysmenorrhea (Spreng 2000b).

Numerous animal studies have documented the programming effects of pre- and post-natal stress on offspring physiology and behaviour, which are remarkably analogous to those induced by foetal glucocorticoid overexposure. Exposing pregnant dams to stressful stimuli, results in both maternal and foetal HPA activation (Williams *et al.* 1999; Hennessy *et al.* 1999). Moreover, these offspring display an activated HPA axis till weaning, and as adults are more anxious and stress-responsive (Takahashi *et al.* 1992). These adverse behavioural effects are associated with altered patterns of brain GR expression, as well as alterations in the circadian rhythm of corticosterone secretion (Koehl *et al.* 1999), consistent with HPA-axis dysregulation. Similarly, prenatal DEX treatment results in permanent life-long hyperactivation of the HPA-axis (Levitt *et al.* 1996), also associated with altered brain GR expression and increased anxiety-related behaviour (Welberg *et al.* 2001). The SNS further participates in stress responses, even in foetal life (McMillen *et al.* 2001), and prenatal exposure to various stressors affects the development of sympathetic innervation and /or its regulation (Young 2002). A recent study on the offspring of rats kept in hypoxia during pregnancy showed altered development of sympathetic centres involved in blood pressure regulation, which continued to adversely affect offspring blood pressure throughout adulthood (Peyronnet *et al.* 2002). In line with these observations, we found that prenatal DEX-treated offspring display hypersensitive pressor responses to even the mildest stressor, and that the resulting stress-induced hypertension is mediated by altered sympathetic nerve responsivity (see Chapter 5). Post-natal manipulations, such as handling new born pups, also induces modified stress responses, by reducing anxiety through altered HPA feedback sensitivity and hippocampal GR expression (Meaney *et al.* 1989; O'Donnell *et al.* 1994).

This chapter reports the phenotypes of offspring treated with either vehicle or DEX during the last week of gestation, which were unintentionally further subjected to pre- and / or post-natal stress, as a result of ultrasonic emission from fluorescent strip lights and / or adjacent construction work. The facility in which our animals are housed was refurbished in 1999, with the installation of a new lighting system, and is located next to

a newly constructed wing of the Western General Hospital, which was completed in summer 2000; the following cohort of animals were both generated and characterised during this time period.

3.2 Methods

3.2.1 Animals

Female rats and their litters were maintained, bred, and administered with vehicle or DEX during their third week of pregnancy as described in Chapter 2. Male offspring were weighed at 3, 7, 14 and 21 days of age, and at three weekly intervals thereafter throughout postnatal life.

At 2 and 3 months of age, male offspring underwent 7 consecutive days of systolic blood pressure measurement by automated tail cuff plethysmography (the last 3 days' measurements were used in subsequent analysis), or 3 days consecutive days of blood pressure measurement by carotid cannulation (where BP was measured in the first 10 minutes after connecting the animal to the apparatus), as described in Chapter 2.

Following the discovery of atypical phenotypes for prenatally treated DEX offspring, adult males, born within the facility, though not subjected to any *in utero* manipulations, were utilised as environmental stress monitors. Following cessation of the construction work, adult naïve males were introduced to the animal facility.

3.2.2 Plasma [corticosterone]

[Corticosterone] in plasma obtained by tail nick sampling was measured in male and female offspring at 3 and 6 months of age, using the method outlined in Chapter 2. Further plasma corticosterone assays were performed on adult males, that were not prenatally treated, but who were born and housed in the animal unit throughout the

generation of this cohort, and in adult naïve males following a month's habituation to the animal unit.

3.2.3 PEPCK activity assay

Measurement of PEPCK activity was made in cytosol derived from homogenates of liver obtained from offspring killed at 3 months of age, according to the methods described in Chapter 2.

3.2.4 Oral glucose tolerance test

Glucose and insulin were assayed in plasma samples from male offspring aged 6 months, collected during the OGTT, using the methods described in Chapter 2.

3.2.5 Assessment of ultrasonic noise pollution

Upon the discovery of atypical phenotypes of prenatally treated animals, a detector used for identifying and locating bats in the wild (kindly donated by the Dept. of Zoology) was set to 80 kHz and used to periodically investigate the animal unit for sources of ultrasound.

3.2.6 Designation of 'Quiet' and 'Noisy' rooms

A motor unit used to power strip lighting within the animals' room was identified as one potential source of ultrasound pollution. This specialised unit enabled the lights to operate a 'dawn' and 'dusk' phase, as opposed to simple 'on/off' function. It was replaced with normal strip lighting in one room, which was termed the 'Quiet' room but left untouched in a second room, which was termed the 'Noisy' room.

3.3 Results

3.3.1 Birth data

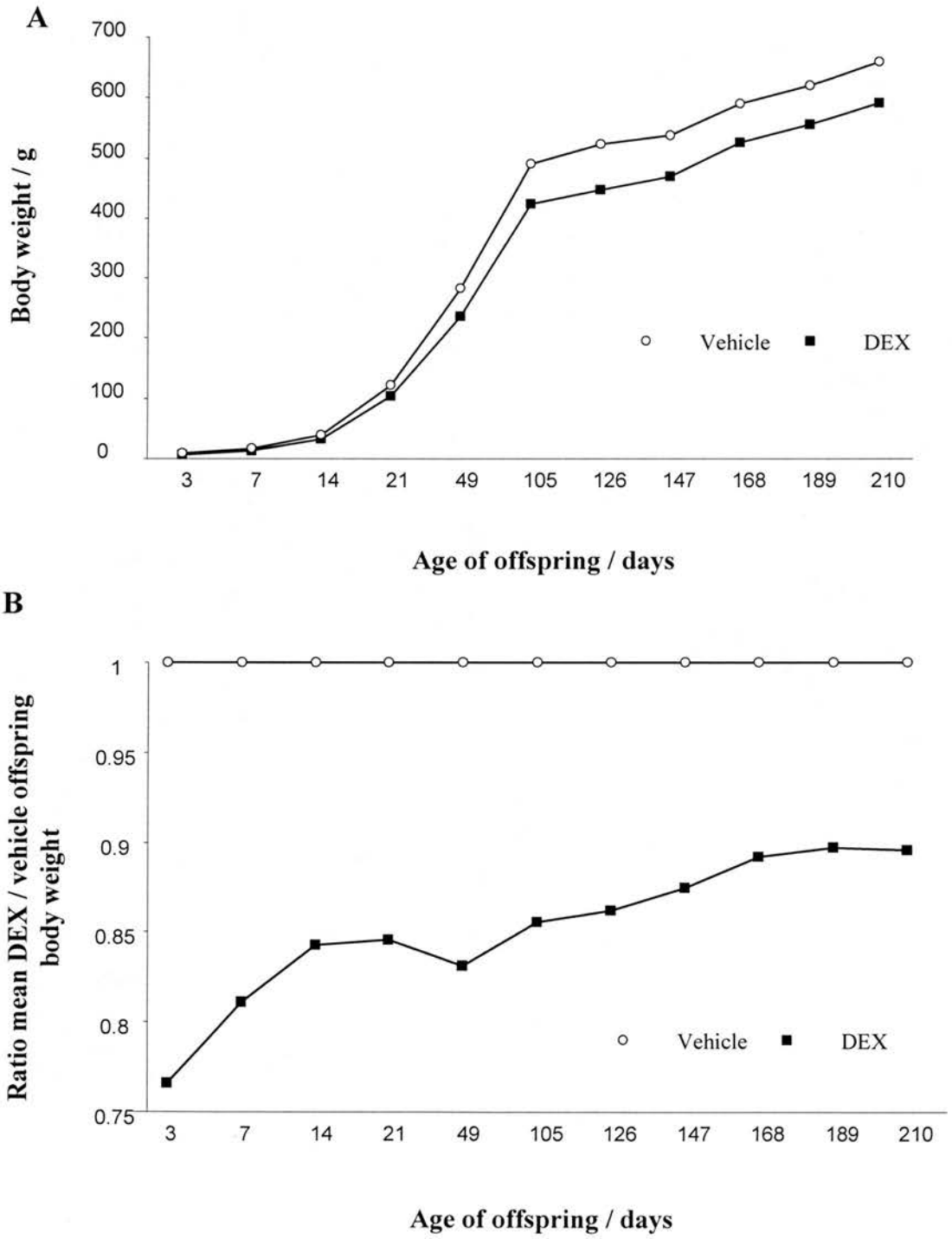
As expected, DEX administration throughout the final week of gestation resulted in a significant decrease in birth weight i.e. vehicle 5.9 ± 0.05g, DEX 5.4 ± 0.05g (n= 67 for both groups, $p < 0.01$ by Student's t-test), representing a mean 8.5% reduction in birth weight as a result of prenatal DEX treatment. No differences in gestation length (Vehicle and DEX; 22 ± 0 days, n = 8 per group) or litter size (Vehicle; 9 ± 1 pups versus DEX; 8 ± 1 pups, n = 67 per group) were noted.

3.3.2 Growth Trajectory

Figure 3-1 reveals that male and female offspring from both treatment groups gained weight throughout the course of their postnatal life. Contrary to many previous studies, DEX-treated offspring only exhibited catch-up growth until approximately 3 months of age, thereafter it ceased and they remained approximately 11% lighter than controls for the remainder of their life; for example, at 210 days of age, mean body masses were 660 ± 14g and 591 ± 12g for vehicle and DEX-treated offspring (n = 34 and 39 respectively, $p < 0.05$ by Student's t-test).

Figure 3-1 Growth trajectory of offspring to 7 months of age

A Growth trajectory of male and female DEX and vehicle treated offspring. Data are presented as mean ± SEM (note the SEMs are contained with the symbols), n = 34 (DEX) and 39 (Vehicle). **B** Catch-up growth of DEX-treated rats: ratio of mean body weights of DEX versus vehicle-treated offspring over the same time period.

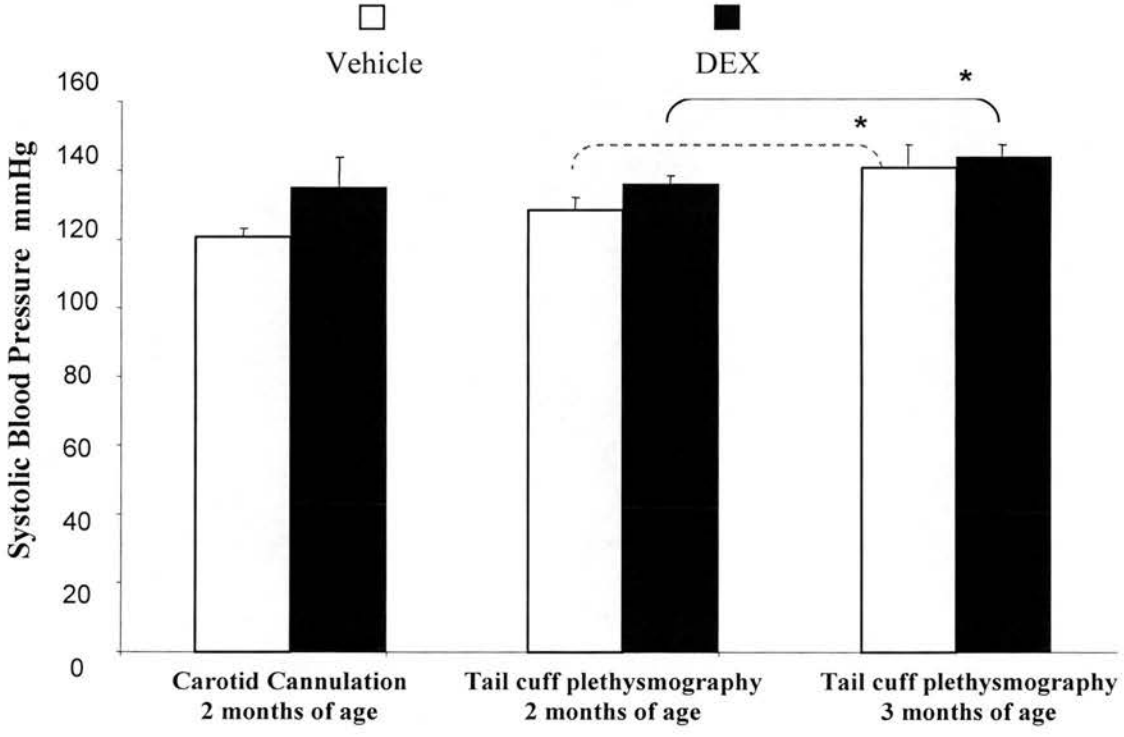


3.3.3 Systolic Blood Pressure

Although there was a trend for DEX-treated offspring to display elevated systolic blood pressure when measured by carotid cannulation, this did not reach statistical significance in the present cohort (see **Figure 3-2**). Similarly, no effect of prenatal treatment was revealed by tail-cuff plethysmography at either 2 or 3 months of age (see **Figure 3-2**). However, systolic blood pressure did increase significantly between 2 and 3 months of age in both vehicle (from 120 ± 7 mmHg to 145 ± 5 mmHg, $p < 0.05$, 1 way ANOVA followed by post-hoc test) and DEX (from 128 ± 2.5 mmHg to 146 ± 5.5 mmHg, $p < 0.05$, 1 way ANOVA followed by post-hoc test) treated offspring.

Figure 3-2 Offspring systolic blood pressure at 2 and 3 months of age

Systolic blood pressure of vehicle and DEX-treated male offspring at 2 and 3 months of age, measured by carotid cannulation (average of 2 consecutive days; readings were taken within the first 10mins after connection of the animal to the apparatus) or tail-cuff plethysmography (average of measurements recorded on the last 3 days of 7 days consecutive measurements). Data are presented as mean ± SEM, n = 4-6 per group for carotid cannulation and n= 10 per group for tail-cuff plethysmography, 1 way ANOVA followed by post-hoc test, * $p < 0.05$.

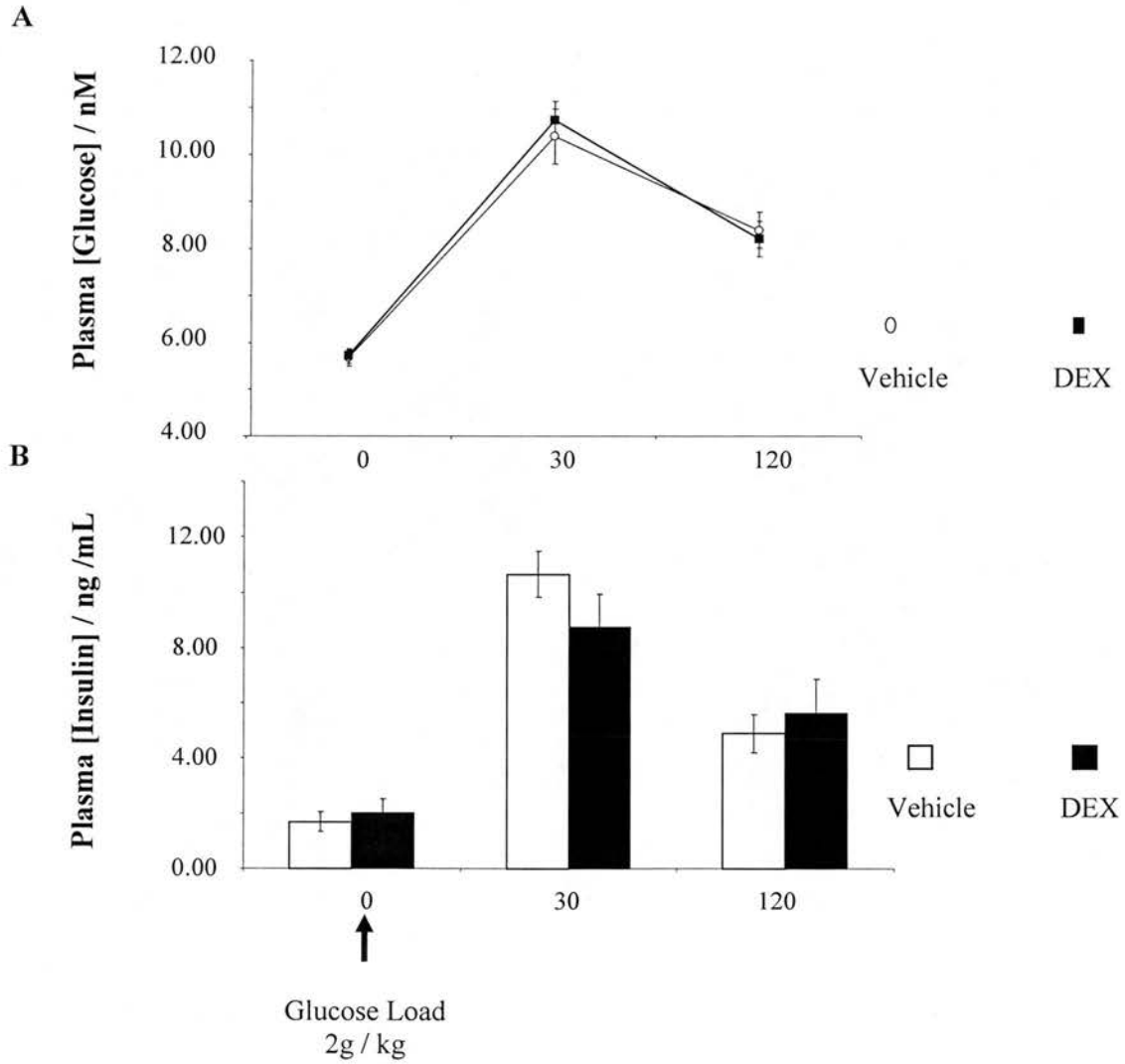


3.3.4 Prenatal DEX and glucose homeostasis

Contrary to previous studies, no difference was observed between the prenatal treatment groups in either plasma glucose (see **Figure 3-3A**) or insulin (see **Figure 3-3B**) during the OGTT, performed at 6 months of age.

Figure 3-3 Plasma glucose and insulin during an oral glucose tolerance test

Plasma [glucose] and [insulin] quantified in blood taken from 09:00 from the tail-tip of rats fasted overnight. **A** Plasma glucose before, and at 30 and 120 mins after an oral glucose load, measured by hexokinase assay. **B** Plasma insulin before, and at 30 and 120 mins after an oral glucose load measured by ELISA. Data are presented as mean ± SEM, n = 7-8 per group.

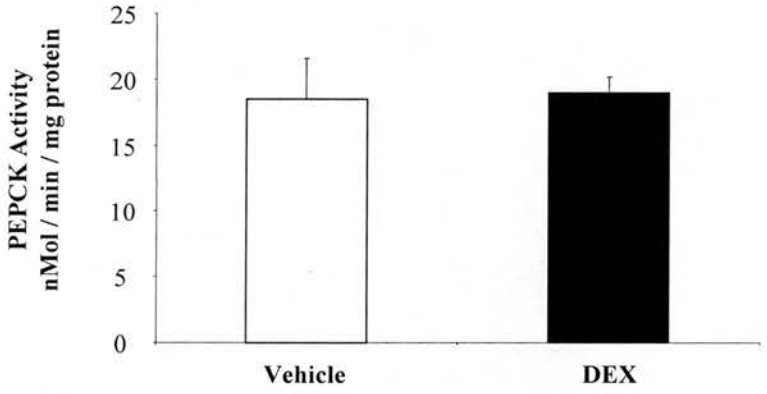


3.3.5 Hepatic PEPCK activity

The lack of effect of prenatal DEX on glucose homeostasis was further associated with a lack of effect of prenatal DEX on liver PEPCK activity, measured at 3 months (see **Figure 3-4**).

Figure 3-4 **Hepatic Phosphoenolpyruvate carboxykinase activity.**

PEPCK activity measured in microsomal extracts from livers of 3 month old vehicle and DEX treated offspring. Data are presented as mean ± SEM, n = 8 per group.

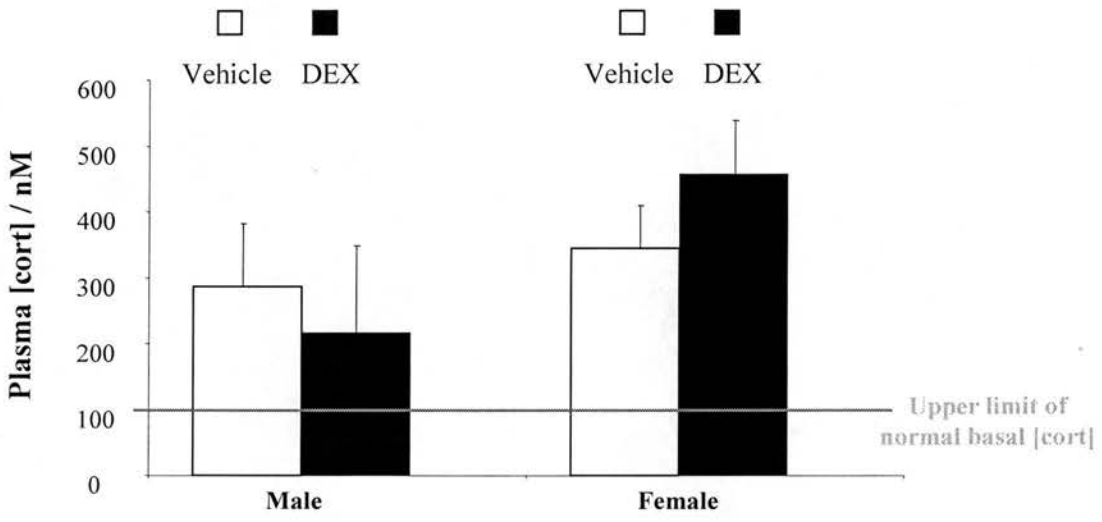


3.3.6 Plasma [corticosterone]

At 3 months of age, both male and female offspring of both prenatal treatment groups displayed greatly elevated increases in basal morning (08:00) plasma CORT (see **Figure 3-6**), compared to levels normally achieved in the laboratory (Levitt *et al.* 1996; Welberg *et al.* 2000; Chapter 4).

Figure 3-5 **Offspring basal plasma corticosterone concentrations**

Plasma [cort] quantified in blood taken from 08:00 from the tail-tip of male and female offspring. Data are presented as mean ± SEM, n = 7-8 per group.



3.3.7 Detection of environmental noise pollution

Considerable ultrasound emissions at 80kHz emanated from the fluorescent strip lighting fitted in the animal rooms of the Biological Research Facility.

It was further noted that the byroad adjacent to the facility was frequently utilised as a route of access for construction lorries destined for the nearby building site. We further learned that the construction of the foundations for the site involved 'piling', a process whereby large steel rods are rammed deep into the ground, resulting in substantial and widespread underground vibrations.

3.3.8 Replacement of lighting and monitoring of corticosterone levels

Following the detection of one potential environmental stressor, the lighting was replaced in one room (termed the 'Quiet' room), and adult males were housed between this room and an adjacent room, where the lighting remained untouched ('Noisy' room). However, measuring basal plasma corticosterone in these animals at four, and six weeks after the lighting was changed revealed no differences between those housed in either the 'Quiet' or 'Noisy' rooms (see **Table 3-1**).

Table 3-1 **Comparison of basal plasma corticosterone obtained in adult males housed in either the 'Quiet' or 'Noisy' rooms**

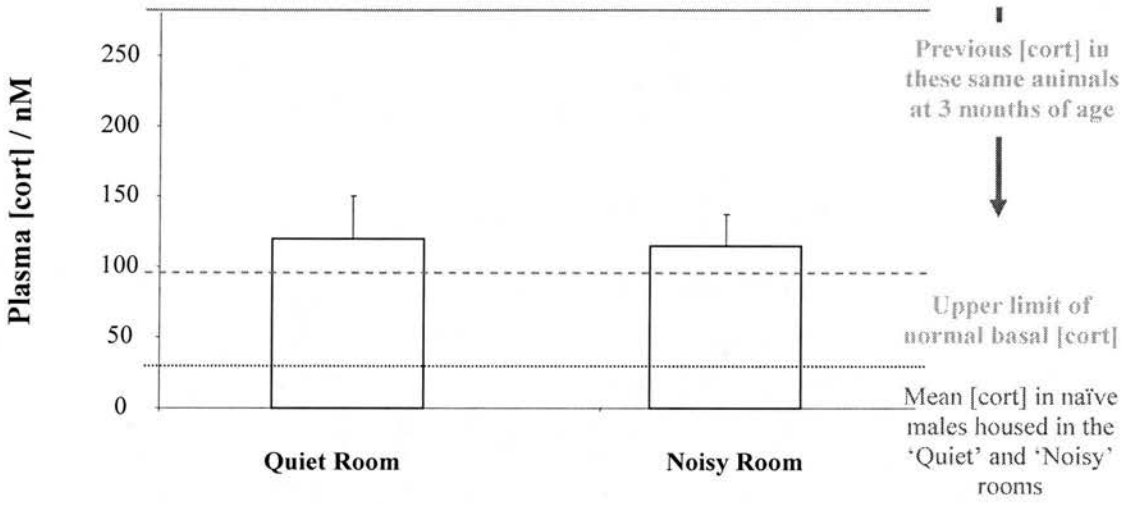
Time of basal corticosterone measurement in adult males	Quiet Room	Noisy Room
4 weeks after the lights were changed	446 ± 158 nM	440 ± 91 nM
6 weeks after the lights were changed	456 ± 142 nM	444 ± 120 nM

3.3.9 Plasma corticosterone levels in vehicle-treated male offspring, and naïve males housed in both 'Quiet' and 'Noisy' rooms.

As demonstrated in **Figure 3-7**, vehicle treated offspring, aged 8 months, housed in both the 'Quiet' and 'Noisy' rooms, revealed similar significant reductions in their basal corticosterone levels, though they still verge on or exceed the upper limit of normal. Conversely, naïve males housed in the 'Quiet' and 'Noisy' rooms had corticosterone levels of 42.5 ± 5 nM, and 31.5 ± 6 nM respectively (N = 8 per room); both values lying within the normal basal range for our laboratory. Construction of the foundations of the new hospital wing was completed at the time of testing.

Figure 3-6 **Corticosterone levels in vehicle-treated male offspring, housed in either the 'Quiet' or 'Noisy' room**

Plasma [cort] quantified in blood taken at 08:00, from the tail-tip of vehicle- treated offspring aged 8 months. Data are presented as mean ± SEM, n = 8 per group. Shown also are the previous mean [cort] for these rats when they were 3 months of age (represented by a solid grey line), the upper limit of normal basal [cort] (represented by a dashed grey line), and the mean [cort] measured in naïve males aged 3 months (represented by a black dotted line). The downward arrow denotes the reduction in [cort] in these animals, which occurred between 3 and 8 months of age.



3.3.10 Comparison of offspring phenotypes pre-, during and post-detection of noise pollution

Characterisation of this cohort revealed a phenotype that differed substantially from previous and subsequent cohorts of prenatally treated offspring. A summary of the results highlighting the discrepancies between offspring cohorts generated for this study, and those before and after the cessation of building work, and modification of the lighting system, is outlined in **Table 3-2**. Finally, offspring cohorts generated simultaneously by Dr. M. Nyirenda at another animal unit elsewhere in the university revealed the previously reported phenotypes for prenatally vehicle and DEX treated offspring (data not shown).

Parameter	Prenatal Treatment	Detection of Noise Pollution		
		Before	***During***	***After***
Birth weight, g	Vehicle	6.63 ± 0.07	5.9 ± 0.05	6.37 ± 0.06
	DEX	5.89 ± 0.15*	5.4 ± 0.05*	5.62 ± 0.07*
		11%* difference *(Levitt et al. '96)*	8.5%* difference	12% * difference *(Chapter 4)*
Basal plasma [cort], nM	Vehicle	79 ± 15	286 ± 96	53 ± 14
	DEX	115 ± 29* *(Levitt et al. '96)*	216 ± 131	139 ± 32* *(Chapter 4)*
Systolic BP, mmHg	Vehicle	133 ± 2.7	141 ± 6	133 ± 3
	DEX	144 ± 2* *(Levit et al. '96)*	144 ± 5	147 ± 3* *(Chapter 4)*
Basal plasma glucose, mM	Vehicle	4.3 ± 0.2	5.67 ± 0.2	
	DEX	5.3 ± 0.3* *(Nyirenda et al. '98)*	5.72 ± 0.4	
Peak glucose in OGTT, mM	Vehicle	7.5 ± 0.2	10.4 ± 0.9	9.3 ± 0.2
	DEX	8.7 ± 0.4* *(Nyirenda et al. '98)*	10.7 ± 0.4	10.6 ± 0.5* *(Dr.M.Cleasby)*
Peak insulin in OGTT, ng / ml	Vehicle	3.8 ± 0.5	10.7 ± 0.8	4.21 ± 0.7
	DEX	6.1 ± 0.5* *(Nyirenda et al. '98)*	8.8 ± 1.2	8.28 ± 1.4* *(Chapter 4)*
Hepatic PEPCK activity	DEX	Increased by 60%* *(Nyirenda et al. '98)*	Increased by 3%	Increased by 40%* *(Chapter 4)*

3.4 Discussion

This chapter recounts data acquired during a series of investigations intended to unravel the molecular mechanisms underlying the hypertensive phenotype of the DEX-programmed rat. Instead, it reveals how the physiological profile of a prenatal vehicle or DEX-treated animal is confounded by perinatal stress, induced in this case by construction noise. Furthermore, during this same time period the animal facility was frequently but irregularly passed by a plethora of construction lorries and machines accessing the adjacent building site, which would have involved the emission of infrasound. Such noise disturbances would have been clearly audible to our cohorts, as rodents possess a hearing range that incorporates both ultra- and subsonic frequencies. Moreover, prenatal DEX-treatment programmes greater cochlear sensitivity to noise stress (Canlon *et al.* 2003), which is most harmful when emitted irregularly and unpredictably (Fride & Weinstock 1984). Thus, these sound frequencies may have induced stress, either pre- and / or postnatally to both vehicle and DEX-treated cohorts through the maternal and / or foetal HPA-axis and / or SNS.

Despite *in utero* growth retardation, the other well documented adult characteristics of the DEX-programmed phenotype were nonexistent. As highlighted by **Table 3-2**, this probably reflects alterations in the vehicle-treated offspring that developed a phenotype analogous to that of DEX-offspring, without any appreciable modification in the phenotype of the latter. Given the null effect of perinatal stress on the phenotype of the DEX-treated offspring, it is tempting to speculate that there is an 'upper limit' for the effects of prenatal glucocorticoids. Whether this 'upper limit' is a result of modified GR and / or MR expression, or is a function of enhanced glucocorticoid metabolism remains to be established. Alternatively, it is conceivable that a factor exists, which antagonises the effects of glucocorticoids, thereby limiting their adverse effects in the long term.

Birth weights of both cohorts, but especially of vehicle-treated offspring were significantly reduced when compared to previous (Levitt *et al.* 1996; Nyirenda *et al.* 1998) and later (see Chapters 4 and 6) studies , implying that the initiation of noise stress occurred before birth. Subsequent catch-up growth of DEX-treated offspring also failed to achieve completeness, and they remained on average 11% lighter throughout adulthood, when compared to controls; though the implications of this are controversial. Whilst complete (Nyirenda *et al.* 1998; Levitt *et al.* 1996; Desai *et al.* 1996; Woodall *et al.* 1996) and lack of catch-up growth (Welberg *et al.* 2001; Cleasby *et al.* 2003; Langley & Jackson 1994) have been demonstrated in several programming models, each reported cohort still developed a 'programmed' phenotype. These studies support the present view that birth weight and therefore, subsequent catch-up growth form relatively crude markers of exposure to an adverse intrauterine environment, rather than underlying a specific cause of pathophysiology in the offspring. In Chapter 4, birth weight and postnatal catch-up growth were observed in both male and female DEX-treated offspring, yet the adult 'programmed' phenotypes differed substantially, consistent with this hypothesis.

At 2 and 3 months of age, no differences in systolic blood pressure were detected by either tail-cuff plethysmography or carotid cannulation. This contrasts with previous reports that utilised these techniques to demonstrate hypertension in DEX-treated adult offspring (Benediktsson *et al.* 1993; Levitt *et al.* 1996; Lindsay *et al.* 1996). Whilst there was an increase in systolic blood pressure between the time of measurement at 2 and 3 months of age, it occurred to the same degree in the offspring of both cohorts, and possibly reflects perinatal stress programming. Interestingly, it is the vehicle-treated offspring that demonstrate blood pressure values equivalent to those normally associated with prenatal DEX-programming (Benediktsson *et al.* 1993; Levitt *et al.* 1996; Lindsay *et al.* 1996), whilst the DEX-treated offspring display typically expected values, again suggesting that perinatal noise stress did not add to their phenotype. Alternatively, it is perhaps tempting to speculate that they in fact resist the effects of stress. In terms of mechanism for the hypertension associated with perinatal stress programming, it is most

likely mediated through elevated glucocorticoids (Levitt *et al.* 1996), altered sympathetic nerve responsivity (Tonkiss *et al.* 1998; Chapter 6), and/ or an activated RAS (Everett *et al.* 1991; Chapter 4). Furthermore, whilst tail-cuff plethysmography and carotid cannulation are associated with considerable stress artifacts, the nature of their varying effects on both vehicle and DEX-treated offspring, does not implicate them as causative factors in the hypertension detected here (Chapter 6).

In further contrast to previous (Nyirenda *et al.* 1998; Nyirenda *et al.* 2001) and subsequent (Chapter 4) findings, prenatal DEX treatment did not affect plasma glucose or insulin levels measured at three time points during an OGTT. However, increases in both these plasma variables following the oral glucose load indicate that nothing went awry with the procedure (Nyirenda *et al.* 1998). In line with birth weight and BP data from the present cohorts, the elevated plasma glucose and insulin concentrations measured in vehicle-treated offspring mirrored those typically expected of prenatal DEX-treated offspring. Furthermore, the comparable insulin-glucose profile of these offspring cohorts is consistent with their identical hepatic PEPCK activities. Such elevated levels of activity are normally associated with the hyperglycaemia found exclusively in prenatal DEX-treated offspring (Nyirenda *et al.* 1998; Nyirenda *et al.* 2001; Chapter 4).

Highly elevated levels of plasma corticosterone, indicative of stress, were observed in both cohort offspring, and again, unlike previous (Levitt *et al.* 1996; Welberg *et al.* 2001) and later (Chapter 4) findings, no differences between cohorts were detected. After the removal of the ultrasonic emitting lights in one room, rat corticosterone levels were assayed at regular intervals, serving as environmental stress monitors. These rats, though born in the animal facility, were not subjected to any *in utero* manipulation. However, they too repeatedly displayed equivalent levels of corticosterone, regardless of which room they were housed in, suggesting that perinatal stress had programmed the HPA-axis of each animal born within the facility, which could be further acted upon by construction noises. Indeed, following the cessation of building works, plasma

corticosterone values fell dramatically, but still remained in excess of the upper normal basal limit, revealing continued life long HPA dysfunction. At this time naïve adult males were introduced to the animal facility, and displayed appropriate basal plasma corticosterone levels, with no differences observed between animals housed in either room, suggesting that exposure to the stressors during the perinatal period is critical to programming later dysfunction. Alternatively, it may suggest that exposure to ultrasonic noise from the strip-lighting alone was insufficient to programme adult dysfunction, and that the phenotypes presented here are a result of the construction disturbances.

In conclusion, it is evident that perinatal stress induced by environmental noise is sufficient to confound prenatal DEX-programming experiments. This perinatal stress successfully introduced low birth weight, hypercorticosteronaemia, insulin resistance and hypertension, to vehicle-treated offspring, such that the typical distinctions between both treatment groups were completely ameliorated. The lack of an additional effect on DEX-treated offspring is suggestive of an 'upper limit' effect of prenatal stress or glucocorticoids, serving to prevent against the potentially detrimental effects of sustained glucocorticoid hyper-exposure. Finally, perinatal stress is further able to programme HPA-axis dysfunction in offspring that were not additionally subjected to any other *in utero* manipulations.

Perspectives

With the demonstrated ability to modify complex physiological systems, it is quite conceivable, that construction work may serve as a major contributor to reports of 'biological variability'. As the following chapters will illustrate, under well-controlled conditions, biological variability is actually quite negligible, and those environmental variables, which may go unnoticed by many researchers (particularly those that utilise animal tissues harvested by others); can cause pronounced, misleading, and costly alterations in otherwise robust model systems.

Glucocorticoid exposure in late gestation permanently programmes gender specific differences in adult cardiovascular and metabolic physiology.

4.1 Introduction

Whilst the precise mechanisms underpinning prenatal programming remain elusive, we hypothesised that excessive fetal exposure to glucocorticoids might be important, based upon the documented effects of antenatal glucocorticoid administration to reduce birth weight and alter the trajectory of maturation of specific fetal organs (see Chapter 1 for more in-depth introduction). In rats, we and others have shown that prenatal exposure to the synthetic glucocorticoid dexamethasone (DEX) or to endogenous maternal glucocorticoids by inhibition of the feto-placental 'barrier enzyme', 11β-hydroxysteroid dehydrogenase type 2, reduces birth weight and produces permanently impaired glucose tolerance and hypertension in the adult offspring, associated with increased hypothalamic-pituitary-adrenal (HPA) axis activity and abnormal behaviour reminiscent of anxiety (Levitt *et al.* 1996; Welberg *et al.* 2000; Welberg *et al.* 2001; Holness & Sugden 2001; Sugden *et al.* 2001). The programming of glucose-insulin dyshomeostasis only occurs when glucocorticoids are administered in the final week of gestation (Nyirenda *et al.* 1998), whereas the programming of hypertension is less time constrained (Levitt *et al.* 1996; Benediktsson *et al.* 1993). Similar findings have been reported with DEX in the sheep (Dodic *et al.* 2001; Gatford *et al.* 2000) and guinea pig (Persson & Jansson 1992; Jansson & Persson 1990; Liu *et al.* 2001), although the particular windows of vulnerability appear species-specific.

In terms of mechanism, whilst prenatal DEX-programmed glucose intolerance is associated with permanently increased hepatic expression of a key gluconeogenic enzyme phosphoenolpyruvate carboxykinase (PEPCK) (Nyirenda *et al.* 1998), the basis

of DEX programming of hypertension remains unknown, although some data in sheep have implicated the renin-angiotensin-aldosterone system (RAS) (Moritz *et al.* 2002; Dodic *et al.* 2002). Importantly, in adult rats, glucocorticoids regulate all of the principle components of the RAS, including renin secretion (Burris *et al.* 1986), angiotensinogen synthesis (Bunnemann *et al.* 1993), angiotensin converting enzyme activity (Mendelsohn *et al.* 1982), angiotensin II (Sato *et al.* 1994) and mineralocorticoid receptor expression (Reul *et al.* 1989). Furthermore, in the fetal rat, hepatic angiotensinogen mRNA levels are regulated by glucocorticoids (Everett *et al.* 1991).

An additional complexity relates to gender. In several paradigms, antenatal manipulations that permanently alter adult HPA axis function are sex-specific (Weinstock *et al.* 1992; Liu *et al.* 2001; Ward & Weisz 1984; Kerchner *et al.* 1995), and the importance of gender in determining programmed outcomes has recently been suggested in both humans (Adair & Cole 2003) and rodents (Ortiz *et al.* 2003; Khan *et al.* 2003). Moreover, there is no a priori reason to assume that if a similar programmed phenotype is obtained that identical mechanisms underlie it. Indeed, early or late gestation DEX or 11β-hydroxysteroid dehydrogenase inhibition all produce HPA axis hyperactivity but involve distinct central mechanisms (Welberg & Seckl 2001; Welberg *et al.* 2001; Welberg *et al.* 2000). However, little work has been done on the effects of antenatal steroids and peripheral programming phenotypes. DEX-programmed hypertension occurs in both sexes, but may reflect distinct processes in each gender, whilst hyperglycaemia/hyperinsulinaemia has only been shown in male rats. This study aimed to determine whether there is sexual dimorphism in the prenatal glucocorticoid programming of (1) glucose intolerance, and (2) hypertension; and (3) to ascertain whether the prenatal programming of adult blood pressure by glucocorticoids is mediated by alterations in the RAS.

4.2 Methods

4.2.1 Animals

Female rats and their litters were maintained, bred, and administered with vehicle or DEX during their third week of pregnancy as described in Chapter 2. Male and female offspring were weighed at 3, 7, 14 and 21 days of age, and at three weekly intervals thereafter throughout postnatal life.

Experiments in which female offspring were utilised, it was ensured that they were all at the same stage of the oestrous cycle, as outlined in Chapter 2.

At 6-7 months of age, offspring underwent 7 consecutive days of systolic blood pressure measurement by automated tail cuff plethysmography, as described in Chapter 2.

4.2.2 HPA activity

Morning and evening corticosterone and ACTH levels was measured in plasma obtained by tail nick sampling from male and female offspring at 3 months of age, using the method outlined in Chapter 2.

4.2.3 PEPCK activity assay

Measurement of PEPCK activity was made in cytosol derived from homogenates of liver obtained from offspring killed at 3 months of age, according to the methods described in Chapter 2.

4.2.4 Oral glucose tolerance test

Glucose and insulin were assayed in plasma samples from offspring aged 6 months, collected during the OGTT, using the methods described in Chapter 2.

4.2.5 Plasma renin activity, angiotensinogen and oestradiol levels

Plasma renin activity (PRA), angiotensinogen and oestradiol levels were measured in the trunk blood of animals aged 8 months, as described in Chapter 2.

4.2.6 Angiotensinogen northern blot hybridisation

Total liver and mesenteric adipose tissue Angiotensinogen (Aogen) mRNA was quantified by Northern blot controlled for by U1 as described in Chapter 2; results are ratios of Aogen:U1 mRNA (arbitrary units;AU).

4.2.7 Real-time PCR

Quantities of hypothalamic and renal Aogen were assessed by real time PCR as outlined in Chapter 2.

4.2.8 Female hepatic GR *in situ* hybridisation

Livers from 5 prenatal vehicle and DEX-treated females were removed after decapitation and fragments quickly frozen on dry ice and stored at -80°C. GR *in situ* hybridisation was performed as described in Chapter 2.

4.2.9 Statistical analyses

All data are expressed as mean ± SEM. Data were compared using an unpaired Student's *t* tests, one-way or multiple ANOVA followed by a LSD post-hoc multiple comparisons test, where appropriate. Values were considered significant when $P < 0.05$

4.3 Results

4.3.1 Gestational weight gain, birth phenotype and catch-up growth

DEX administration throughout the final week of gestation significantly reduced maternal weight gain (see **Table 4-1**). There were no differences in the length of gestation, litter size, ratio of male to female pups born or pup viability. DEX treatment reduced the birth weight of offspring, similarly in both male and female pups (see **Table 4-1**).

At weaning (postnatal day 21) and throughout adulthood, body weights of dexamethasone and vehicle-exposed offspring were similar, both in males and in females, *i.e.* full catch-up growth had occurred (see **Table 4-2**).

Table 4-1 Comparison of gestational and birth parameters in vehicle and DEX treated cohorts

Maternal weight before treatment, maternal weight gain during the last week of pregnancy (days 14-21), gestation length, litter size, male: female ratio, and group, male, and female offspring birth weight. Results are mean ± S.E.M; Student's t-test, * $p < 0.05$, † $p < 0.01$ compared with vehicle group.

	Maternal weight before treatment (g)	Maternal weight gain (g)	Gestation length (d)	Litter size	Male:female ratio	Birth weight Group	Male (g)	Female
Vehicle	372 ± 14 (n = 8)	94 ± 5	22 ± 0	13 ±1.6	0.9 ± 0.1	6.4 ± 0.06 (n = 107)	6.5 ± 0.08 (n = 58)	6.2 ± 0.08 (n = 49)
DEX	373 ± 6 (n = 9)	55 ± 4*	22 ± 0	12 ± 1.2	1.2 ± 0.2	5.6 ± 0.06† (n = 112)	5.7 ± 0.09† (n = 55)	5.6 ± 0.09† (n = 57)

Table 4-2 Comparison of post-natal growth in vehicle and DEX treated offspring

Offspring body weights (grams) of pregnant rats treated with vehicle (control) or DEX during the last week of gestation (embryonic days 14-21), measured at weaning (postnatal day 21), 3 months, and 6 months. Data are presented as mean ± SEM.

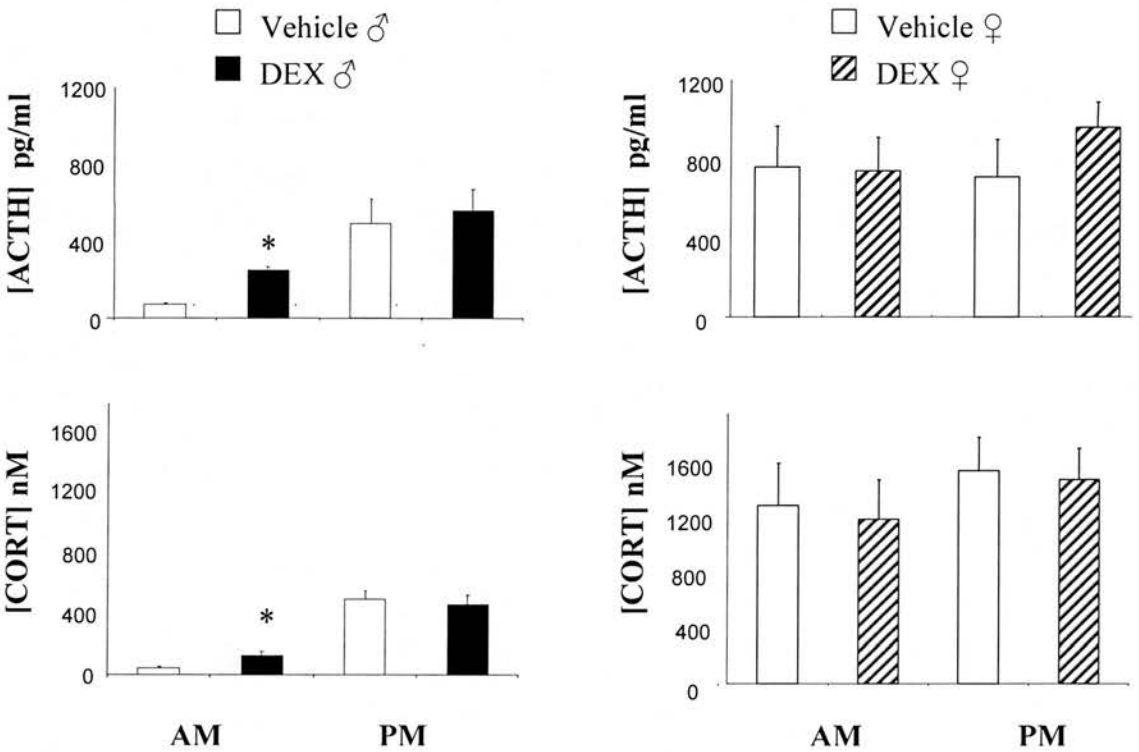
	Weight at weaning (g)	Weight at 3 months (g)	Weight at 6 months (g)
Vehicle			
Group	56.4 ± 1.7 (n = 51)	390 ± 8.6 (n = 34)	532.9 ± 10.0 (n = 34)
Male	57.6 ± 1.1 (n = 26)	493 ± 9.9 (n = 20)	693.9 ± 12.2 (n = 20)
Female	55.7 ± 1.0 (n = 25)	287 ± 7.2 (n = 14)	372.0 ± 7.8 (n = 14)
DEX			
Group	54.0 ± 0.9 (n = 56)	382 ± 11.5 (n = 38)	517.9 ± 16.1 (n = 38)
Male	55.1 ± 1.2 (n = 29)	480 ± 11.6 (n = 20)	674.8 ± 22.3 (n = 20)
Female	52.7 ± 1.4 (n = 27)	283 ± 11.5 (n = 18)	361.0 ± 9.9 (n = 18)

4.3.2 HPA activity in adult offspring

In male offspring, prenatal DEX exposure resulted in significant increases in basal morning (08:00) plasma ACTH and corticosterone concentrations, whereas levels at the evening, diurnal peak, (20:00) were unchanged (see **Figure 4-1**). These hormonal profiles were not reflected in female offspring treated with DEX, with no differences observed between either group at either time point (see **Figure 4-1**). Overall, female offspring displayed significantly higher basal and peak ACTH and corticosterone concentrations.

Figure 4-1 HPA activity in offspring

Basal (AM) and peak (PM) adrenocorticotrophin (ACTH) and corticosterone (CORT) levels in male and female offspring that have been exposed to either vehicle or DEX during the last week of intrauterine life. Results are mean ± S.E.M, 2-way ANOVA (prenatal treatment x time), $n = 8$ per group, * $p < 0.05$ compared with vehicle group.



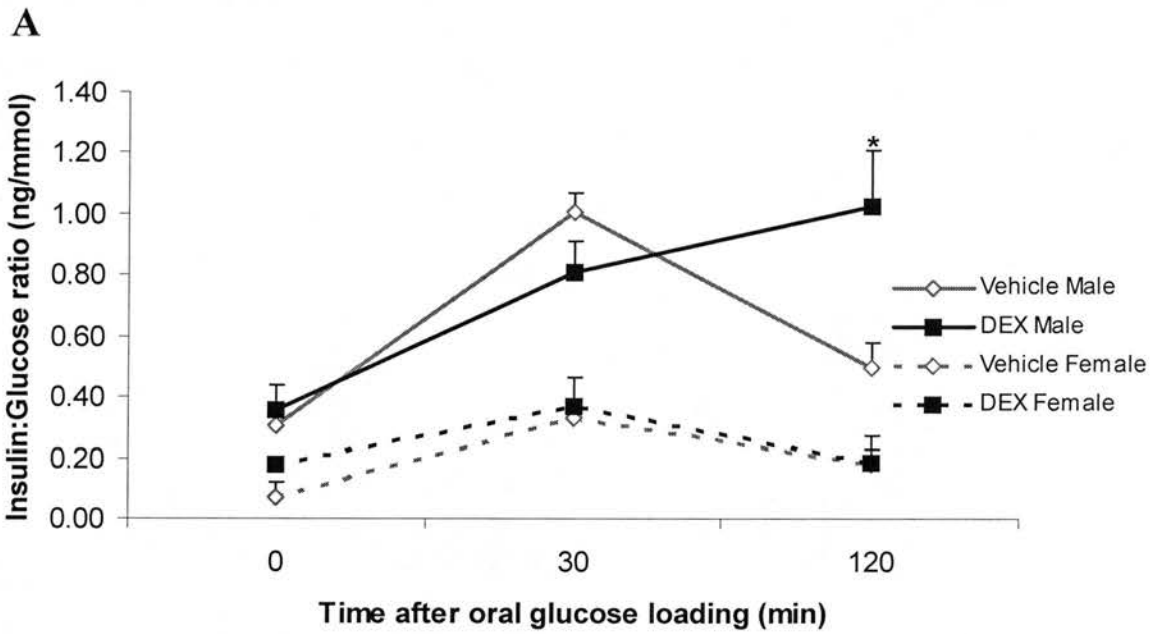
4.3.3 Prenatal DEX, glucose homeostasis and PEPCK activity

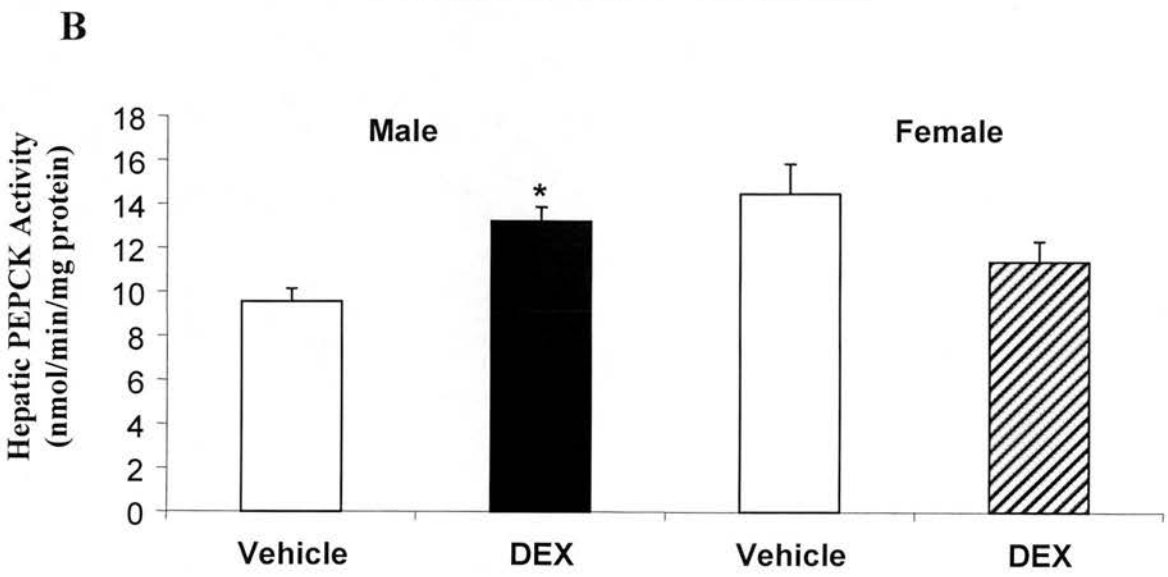
Male offspring of dams treated with DEX throughout the last week of pregnancy had permanent reactive hyperglycaemia and hyperinsulinaemia 120 min after an oral glucose load (see **Figure 4-2A**). In contrast, their prenatal DEX-exposed female offspring littermates did not differ from controls in their glucose homeostasis at any time point pre-, or post-oral glucose load (see **Figure 4-2A**).

Consistent with previous observations (Nyirenda *et al.* 1998), prenatal DEX exposed male offspring had significantly elevated hepatic PEPCK activity, whilst no differences in enzyme activity were observed in their female littermates (see **Figure 4-2B**).

Figure 4-2 Plasma glucose and insulin during an oral glucose tolerance test, and hepatic phosphoenolpyruvate carboxykinase activity.

A. Insulin: glucose ratios in male and female adult offspring, exposed to vehicle or DEX during the final week of pregnancy (embryonic days 14-21). Following an overnight fast, offspring underwent an oral glucose tolerance test. Plasma glucose and insulin was measured at 0, 30 and 120 after an oral glucose load ($2g.kg^{-1}$). **B.** Activity of hepatic PEPCK, assayed at weaning (postnatal day 21) in fed male and female offspring treated *in utero* with vehicle or DEX during the third week of gestation. All results are mean ± S.E.M, **A.** 2-way ANOVA (prenatal treatment x gender), **B.** 3-way ANOVA (prenatal treatment x gender x time), n = 7-8 per group, * $p < 0.05$ compared with vehicle group.



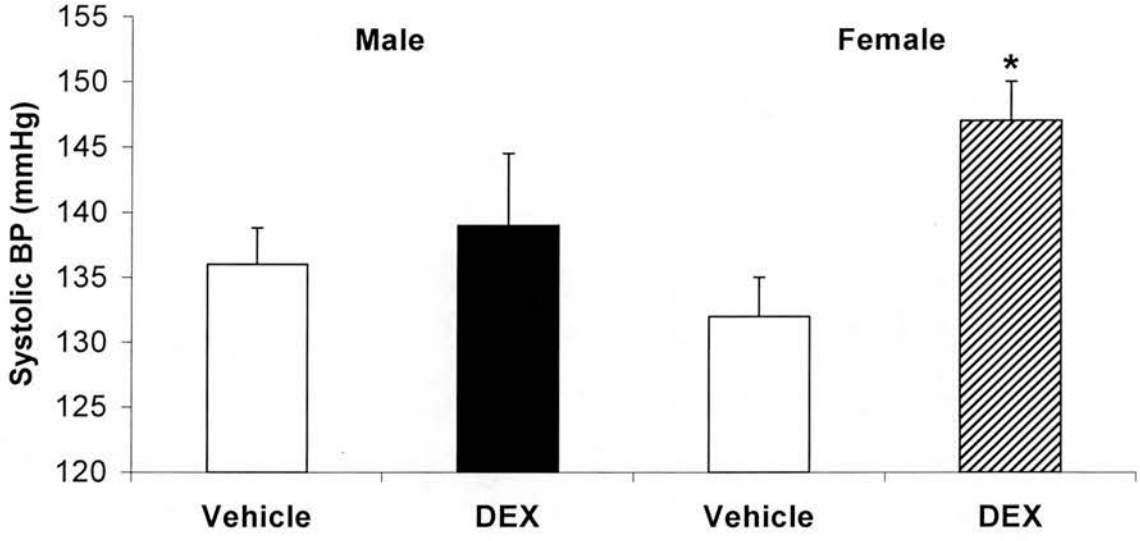


4.3.4 Systolic Blood Pressure

As shown in **Figure 4-3**, only female offspring in the prenatal DEX group showed elevated systolic BP above vehicle controls, when measured at 6 months by tail-cuff plethysmography. In this cohort of prenatally DEX treated animals, adult male offspring systolic BP was not elevated.

Figure 4-3 Offspring systolic blood pressure at 6-7 months of age

Systolic blood pressures, measured by tail-cuff plethysmography, in adult male and female offspring exposed to vehicle or DEX throughout the final week of intrauterine life (embryonic days 14-21). Results are mean ± S.E.M, $n = 8$ per group, * $p < 0.05$ compared with vehicle group.



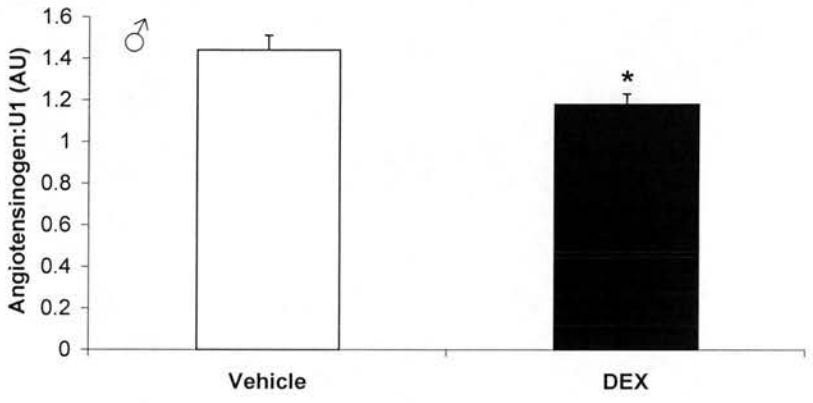
4.3.5 Prenatal DEX and tissue Angiotensinogen expression

Prenatal DEX treated female offspring displayed a significant increase in hepatic angiotensinogen mRNA expression (see **Figure 4-4B**) in association with the hypertension. In contrast, DEX exposed males, which were not hypertensive, had significantly decreased hepatic angiotensinogen mRNA expression (see **Figure 4-4A**). The effect on angiotensinogen mRNA in liver was specific to this tissue since expression of this transcript in female or male mesenteric adipose tissue, kidney and hypothalamus did not reveal any significant differences (see **Figure 4-5**). However, renal angiotensinogen mRNA levels were higher in females (see **Figure 4-5A**), consistent with previous reports (Gordon *et al.* 1992).

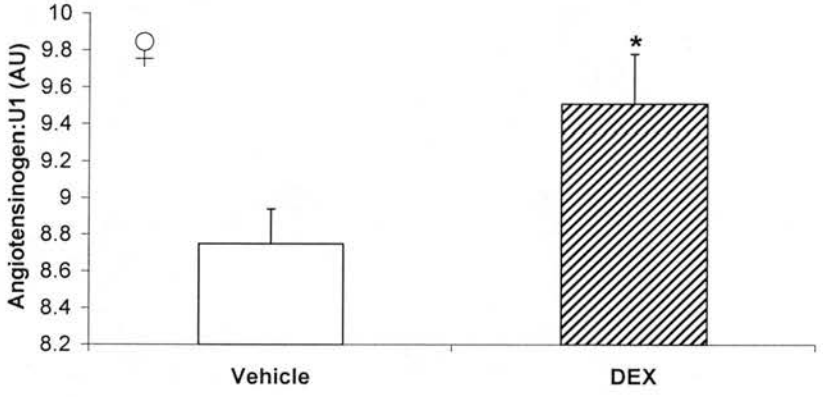
Figure 4.4 Offspring levels of hepatic Angiotensinogen mRNA expression

Hepatic expression of angiotensinogen mRNA, in 8 month old male (graph **A**) and female (graph **B**) offspring, treated with vehicle or DEX during the last week of gestation (embryonic days 14-21). Data from Northern blots, graphically represented in arbitrary units (AU) of angiotensinogen mRNA in adult rat liver. Values shown are normalised to U1 used as loading control. Results are mean ± S.E.M, Student's t-test, n = 6 per group, * $p < 0.05$ compared with vehicle group. **C.** Example of a typical Northern blot, showing distinct angiotensinogen bands.

A.



B.



C.

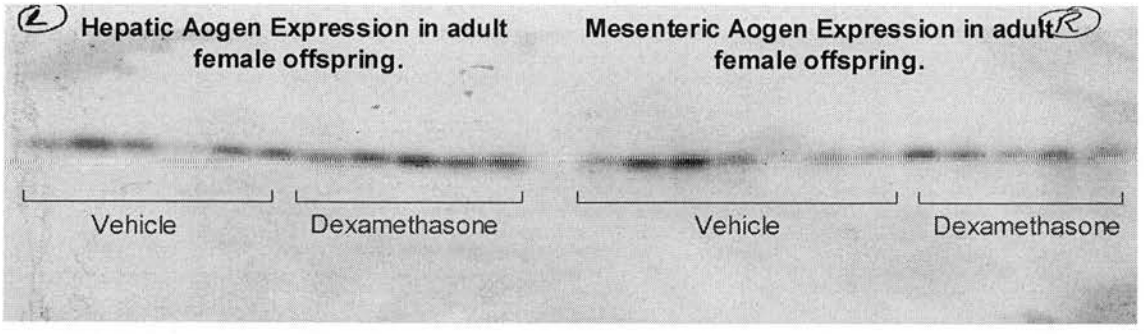
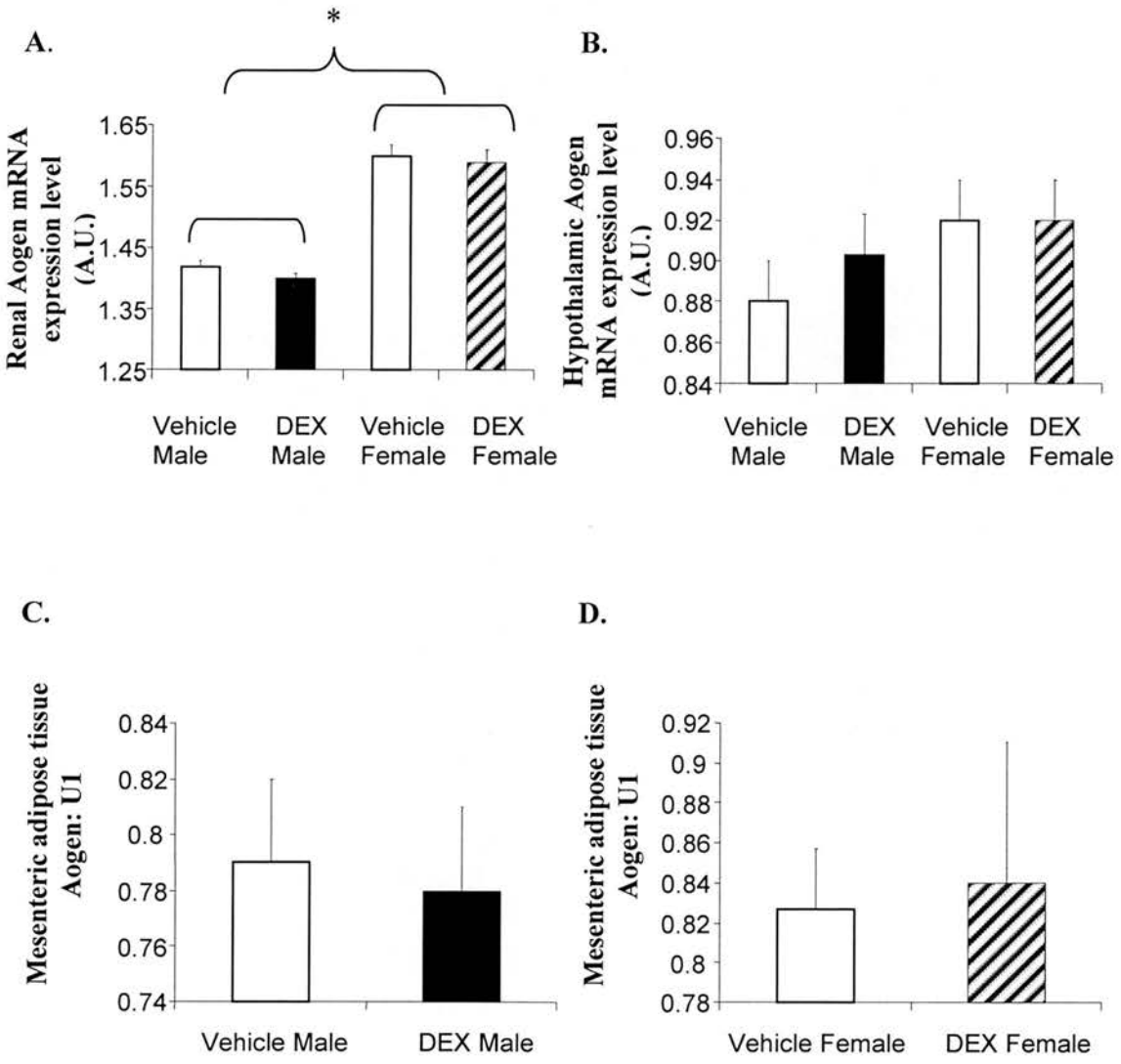


Figure 4-5 Offspring levels of renal, hypothalamic and mesenteric adipose Angiotensinogen mRNA expression

Renal (graph **A**), hypothalamic (graph **B**) and mesenteric adipose tissue (graphs **C** and **D**) expression of angiotensinogen (Aogen) mRNA, in adult male and female offspring, treated with vehicle or DEX during the last week of gestation (embryonic days 14-21). Data from real-time PCR (graphs **A** and **B**), and Northern blots (graphs **C** and **D**), graphically represented in arbitrary units (AU) of angiotensinogen mRNA in adult tissue. Values shown for Northern blots are normalised to U1 used as loading control. Results are mean ± S.E.M, 2-way ANOVA (prenatal treatment x gender) (graphs **A** and **B**) and Student's t-test (graphs **C** and **D**), $n = 6$ per group, * $p < 0.05$ compared with vehicle group.



4.3.6 Plasma angiotensinogen, renin activity and oestradiol parameters

To determine whether the increased hepatic angiotensinogen mRNA levels in prenatal DEX-exposed female offspring were reflected in increased circulating RAS activities, plasma renin activity and angiotensinogen levels were measured in 6-7 month old offspring. Prenatal DEX-exposed female offspring displayed significantly elevated levels of both plasma angiotensinogen and renin activity (see **Table 4-3**). Whilst angiotensinogen is regulated by sex steroids as well as glucocorticoids, no differences in plasma oestradiol levels between DEX and control offspring were found (see **Table 4-3**). Levels of angiotensinogen and renin activity were similar to control in male DEX offspring (see **Table 4-3**).

Table 4-3 Offspring plasma renin activity, angiotensinogen and oestradiol levels.

Adult plasma renin activity, angiotensinogen and oestradiol. Measurements were made in *ad-libitum*-fed, 6-7 month-old male and female rat offspring, treated *in utero* during the last week of pregnancy (embryonic days 14-21) with vehicle or DEX. Results are mean ± S.E.M, $n = 6$-8 per group, 2-way ANOVA (prenatal treatment x gender) and Student's t-test (for oestradiol), * $p < 0.05$ compared with vehicle group.

Plasma Parameter	Male		Female	
	Vehicle	DEX	Vehicle	DEX
Renin Activity (ng.h^{-1}.ml^{-1})	1205 ± 129	1494 ± 169	792 ± 86	1493 ± 238*
Angiotensinogen (pg.µl^{-1})	1686 ± 243	1501 ± 61	482 ± 59	775 ± 103*
Oestradiol (pg.ml^{-1})			18 ± 4	20 ± 4

4.3.7 Effect of prenatal DEX on hepatic GR expression in female offspring

Consistent with DEX-treated male offspring (Nyirenda *et al.* 1998), GR mRNA was significantly increased in the liver of DEX-treated female offspring (Vehicle, 158 ± 51 estimated grains per hepatocyte versus DEX, 317 ± 44 estimated grains per hepatocyte; n = 5 per group, Student's t-test, *$p < 0.05$).

4.2 Discussion

Data presented in this chapter demonstrate that DEX administration in the last week of gestation reduces offspring birth weight and programmes adult cardiovascular and metabolic physiology in a sex specific manner. In male offspring, prenatal glucocorticoid exposure programmes elevated basal circulating corticosterone, elevated hepatic PEPCK activity, and produces adulthood postglucose hyperglycaemia and hyperinsulinaemia. In female offspring, prenatal dexamethasone treatment programmes elevated hepatic angiotensinogen mRNA expression, elevated plasma angiotensinogen and renin activity, and produces hypertension.

Birth weight reduction and postnatal catch-up growth were observed in both males and females in this study, whereas the adult 'programmed' phenotypes differed considerably. This reinforces the current notion that birth weight is merely a relatively crude marker of exposure to an adverse (late) gestational environment, rather than a specific cause of pathophysiology in the offspring. Below specific aspects of the adult phenotype are discussed.

HPA axis

As with some other antenatal challenges (stress, alcohol, etc), antenatal DEX exerts sex-specific effects on the adult HPA axis. In males, last trimester DEX selectively increases morning basal ACTH and corticosterone levels, findings in agreement with earlier studies, which further showed decreased levels of the glucocorticoid and mineralocorticoid receptors in the hippocampus, suggesting decreased HPA axis feedback sensitivity (Levitt *et al.* 1996; Welberg *et al.* 2001). Female HPA function was not altered by DEX. Two possible explanations occur; either females resist DEX programming or the 'stress' of antenatal vehicle injections was sufficient to programme the offspring HPA axis to the same extent as DEX in females. In support of the latter explanation, many sexually dimorphic antenatal programming paradigms affecting the HPA axis in rats are more marked in females (Weinstock *et al.* 1992; Osborn *et al.*

1996), though this is by no means consistent (Kim *et al.* 1999; Koehl *et al.* 1999). Moreover, female, but not male offspring rat HPA parameters can be programmed by vehicle injections to a similar degree as last trimester DEX (Bakker *et al.* 1998). Moreover, basal corticosterone and ACTH levels in the antenatal vehicle-injected females were higher than seen in uninjected control females in our laboratory (personal communication, Dr. Amanda Drake). In contrast, some data support different trajectories for the development of HPA axis components in the rat, suggesting last trimester DEX may impact upon different tissue sensitivity windows in males and females (Aird *et al.* 1997). In the guinea pig, similar sex specific programming of the HPA axis is also observed (Liu *et al.* 2001). Whether CNS glucocorticoid receptor density is altered in female DEX offspring remains the target of future studies. Nevertheless, the sex difference in HPA axis programming by DEX is clear-cut.

Glucose-Insulin homeostasis

In agreement with previous observations (Nyirenda *et al.* 1998; Nyirenda *et al.* 2001), DEX-treated male offspring were hyperglycaemic following an oral glucose load with features suggestive of insulin resistance (higher 120 min insulin-to-glucose ratio). As before, there was a permanent increase in hepatic PEPCK activity. None of these effects were seen in female littermates. Similar gender-specific programming of insulin resistance has been observed in the male offspring of protein restricted dams (Sugden & Holness 2002), and the 2 antenatal challenges may be related as the diet reduces placental 11β-HSD2 allowing excess fetal glucocorticoid exposure (Langley-Evans *et al.* 1996). It is tempting to speculate that the gender difference reflects the lack of HPA programming in females. In support of this contention, raised hepatic PEPCK in males is associated with permanently increased liver GR expression and hence increased sensitivity of plasma glucose to circulating corticosterone levels, which are elevated in males. Furthermore, this would explain why increased hepatic GR expression was not associated with raised hepatic PEPCK activity in DEX-treated females. Alternatively, it could be that insulin resistance (absent in the DEX-treated females) is necessary before glucocorticoids can affect PEPCK activity (Reshef *et al.* 1970; Friedman *et al.* 1993).

However, the control of PEPCK gene expression is complex and is only one part of the myriad processes regulating glucose homeostasis. Alternative explanations, including the role of oestradiol (though unaltered in this study), thyroid hormones and insulin (Hall *et al.* 2000; Lucas *et al.* 1991), in modulating hepatic PEPCK and other genes, any of which may be differentially altered in males and females, remain to be addressed. Nonetheless, the parallels between programming (or not) of HPA hyperactivity and of glucose dyshomeostasis remain intriguing.

Blood pressure

Surprisingly, in the current study elevated systolic blood pressure was confined to DEX-treated female offspring. Prior investigations have demonstrated higher blood pressures in DEX-treated males, though there were differences in the prenatal treatment regime (Benediktsson *et al.* 1993) and measurement technique used (Benediktsson *et al.* 1993; Levitt *et al.* 1996). Interestingly, there is a suggestion that the timing of blood pressure measurement may be important. Exposing dams to DEX, Ortiz et al found elevated systolic blood pressure specifically confined to female offspring at 2 months of age, and to male offspring at 6 months (Ortiz *et al.* 2003). In another study, the female offspring of lard-fed dams did not reveal any elevation in blood pressure at 3 months of age but did at 6 and 12 months, whilst the male offspring were normotensive at the selected time points (Khan *et al.* 2003). Here offspring blood pressure was assessed at 6-7 months of age, whereas our previous studies demonstrating hypertension in DEX-treated males used younger animals (Benediktsson *et al.* 1993; Levitt *et al.* 1996). The reasons for the disparity in these programming models are unknown.

Whatever the basis for the sex difference in this study, prenatal glucocorticoid administration resulted in generalised activation of the RAS in the hypertensive female but not the normotensive male littermate offspring. In rodents, angiotensinogen is a key step determining RAS activation (Tamura *et al.* 1996) and it is a strong candidate gene for hypertension in humans (Corvol *et al.* 1999). Thus, the higher hepatic angiotensinogen mRNA levels and consequent elevation in plasma angiotensinogen

concentrations in females may drive the hypertension. Non-hepatic sources of angiotensinogen were not altered, emphasising the liver-specificity of this effect, (as on other gene expression in this model; Nyirenda *et al.* 1998), in contrast to suggestions in the sheep (Dodic *et al.* 2001). Moreover, their accompanying increase in PRA, is consistent with the current notion that angiotensinogen is not merely a passive substrate reservoir (Bohlender *et al.* 2000). Recent evidence suggests that the renin-angiotensinogen complex acts to stabilise plasma renin concentrations (by increasing renin's half life and decreasing its metabolic clearance rate), thereby regulating plasma renin independently of renal renin secretion and AngII mediated feedback (Bohlender *et al.* 2000). Thus, increased plasma angiotensinogen and PRA may co-exist to promote hypertension. In contrast, prenatally DEX-treated males showed reduced hepatic angiotensinogen mRNA levels, and unaltered plasma angiotensinogen and PRA. Importantly, circulating oestradiol did not vary physiologically between the groups of prenatally treated female offspring, though the naturally higher female levels could in part underlie the gender differences in RAS programming.

In conclusion, prenatal DEX administration in the final week of gestation, results in reduced birth weight, and subsequent gender specific abnormalities in cardiovascular and metabolic physiology. Alterations within the RAS may, at least in part, underlie the hypertension. Recent human data in preterm babies have suggested that brief prenatal glucocorticoid treatment, commonly used in obstetric practice, is associated with increased adolescent blood pressure (Doyle *et al.* 1999). These experimental observations, in conjunction with others, highlight the necessity for meticulous follow-up studies to ascertain whether antenatal glucocorticoid exposure in humans produces these adverse effects, and to further determine whether they are gender specific.

Prenatal dexamethasone, dietary sodium and hypertension.

5.1 Introduction

Considerable research on the effects of dietary sodium on hypertension has generated a wealth of contentious data, and as the debate ensues, one key question remains to the fore; "how much to use in a healthful manner?" Conventional philosophy promotes the beneficial blood pressure lowering effects of a low sodium diet; though its supporters frequently cite studies utilising rodents that are predisposed to developing hypertension when fed a sodium diet far in excess of normal nutritional requirements (Rapp 1982; Ely 1997). Moreover, the effectiveness of a low sodium diet is highly dependent on both the stage of development and strain of rat used (Di Nicolantonio *et al.* 1990). Whilst these very low sodium diets may reduce blood pressure (Ely *et al.* 1985), they are associated with adverse effects such as hyperlipidaemia and insulin resistance (Fliser *et al.* 1995). Furthermore, in certain experimental models, a low sodium diet actually increases blood pressure: in uninephrectomised rats (Seymour *et al.* 1980) and those fed a low sodium diet during weaning (Webb *et al.* 1987). As prenatal DEX has been demonstrated to decrease nephron number (Oritz *et al.* 2002), this could possibly reduce renal function thereby mimicking uninephrectomy. Most interestingly, low sodium diets have recently been identified as programmers of low birth weight, and of hypertension in later life (Roy-Clavel *et al.* 1999).

The mechanisms by which a low sodium diet may produce hypertension remain to be fully elucidated, though alterations in vascular reactivity (Rossiter *et al.* 1995), renal function and the RAS (Battista *et al.* 2002) have been implicated.

To our knowledge, no previous study has examined the effect of a low sodium diet on the cardiovascular and metabolic physiology of a prenatally glucocorticoid programmed

rat. Intriguingly, in one study, rats receiving daily injections of DEX and fed a sodium deficient diet revealed significant increases in aldosterone synthase mRNA expression when compared to those receiving: high salt, low salt, DEX, or DEX and a high salt diet (Malee & Mellon 1991). With this in mind, we hypothesised that prenatal DEX would result in a similar increase in aldosterone synthase gene expression when offspring were fed a low sodium diet, thereby resulting in a physiological increase in aldosterone and blood pressure.

5.2 Methods

5.2.1 Animals

Female offspring from both prenatal vehicle and DEX-treated dams were chosen from the offspring cohorts generated in Chapter 4.

At 6 months of age, 12 female offspring from both cohorts were selected at random and underwent 7 days of consecutive blood pressure measurement by tail-cuff plethysmography, as outlined in Chapter 2.

At the end of the week, 6 females from both cohorts were placed on either a normal (0.3%) or low (0.03%) sodium containing diet. Offspring were maintained on the diet for 14 days, and had their blood pressure measured by tail-cuff plethysmography every third day for the first nine days, as outlined in Chapter 2.

5.2.2 Oral glucose tolerance test

11 days after initiation of the study, glucose and insulin were assayed in plasma samples from offspring, collected during the OGTT, using the methods described in Chapter 2.

5.2.3 Plasma renin activity, aldosterone and corticosterone levels

At the end of the study (day 14), renin activity, aldosterone and corticosterone were assayed in plasma samples from offspring, collected from trunk blood during sacrifice, as outlined in Chapter 2.

5.2.4 Re-evaluation of dietary sodium content

Due to the aberrant nature of the results generated throughout the course of this study, the sodium content of the diets were re-assessed both independently by the Dept. of Clinical Chemistry at Edinburgh's Western General Hospital, and by Special Diet Services, the company which supplied the diets.

5.3 Results

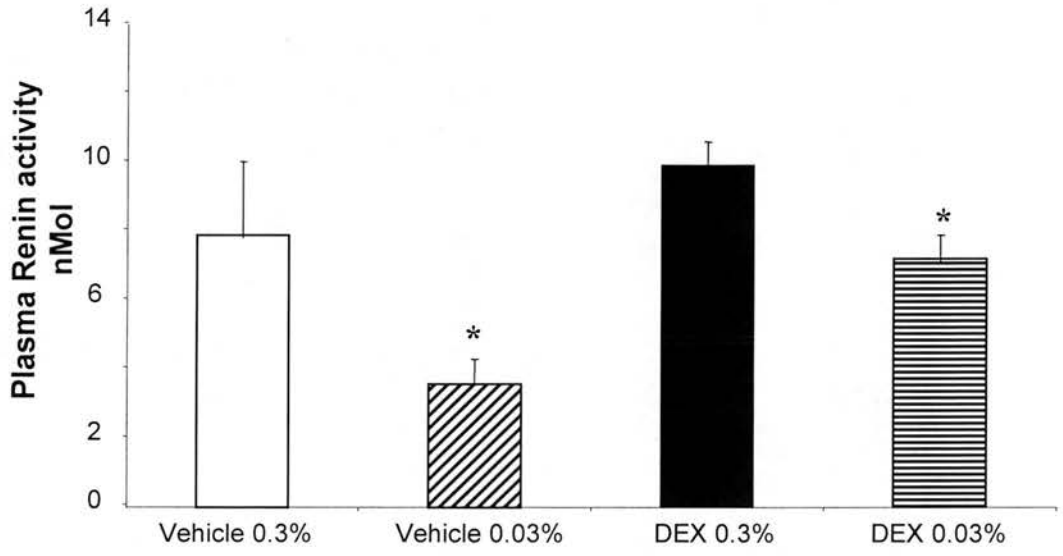
5.3.1 Effect of dietary sodium on plasma renin activity, and plasma aldosterone

A plasma renin activity (PRA) assay was performed 14 days after offspring had been started on the sodium diets. As revealed by **Figure 5-1**, offspring fed the low sodium diet had significantly *lower* PRA values; an absolutely counterintuitive finding given that lower dietary sodium should increase PRA.

Furthermore, in association with lower PRA levels, those offspring fed a low sodium diet also had lower, albeit not significant, plasma aldosterone levels, (normal sodium diet: vehicle, 251 ± 32 pmol / l versus DEX, 267 ± 47 pmol / l; low sodium diet: vehicle, 211 ± 19 pmol / l versus DEX, 218 ± 24 pmol / l; 2-way ANOVA (prenatal treatment x diet), $p = 0.5$ compared with vehicle group).

Figure 5-1 Effect of dietary sodium on plasma renin activity

Renin values were assayed in trunk plasma, collected at sacrifice in 6 month old female vehicle and DEX-treated offspring, following 14 days on either a normal (0.3%) or low (0.03%) sodium diet. All results are mean ± SEM, Student's t-test. n = 6 per cohort, * $p < 0.05$ compared to normal sodium diet.



5.3.2 Multiple re-analyses of dietary sodium content

Owing to the unexpected and counterintuitive PRA and aldosterone data, we decided to re-evaluate the sodium content of the supplied diets. This was achieved both independently by the Dept. of Clinical Chemistry, at the Western General Hospital, and by returning some of the diets for re-analysis to Special Diet Services, the company which supplied them to us. As **Table 5-1** illustrates, there was an alarming disparity between the dietary sodium content requested and those actually supplied and utilised experimentally. Each re-evaluation concurred that the supposed 'normal 0.3%' sodium containing diet actually contained sodium in the region of 0.09%, over 3 times less than desired. Moreover, the supposed 'low 0.03%' sodium containing diet had more than 12 times the requested sodium content, implying the diet was more saline than standard chow.

Table 5-1 Re-evaluation of dietary sodium content

Following confounding experimental results, the sodium content of the diets utilised throughout the course of the current study were re-analysed; both independently by the Dept. of Clinical Chemistry, at Edinburgh's Western General Hospital and by Special Diet Services, the company which originally supplied the diets.

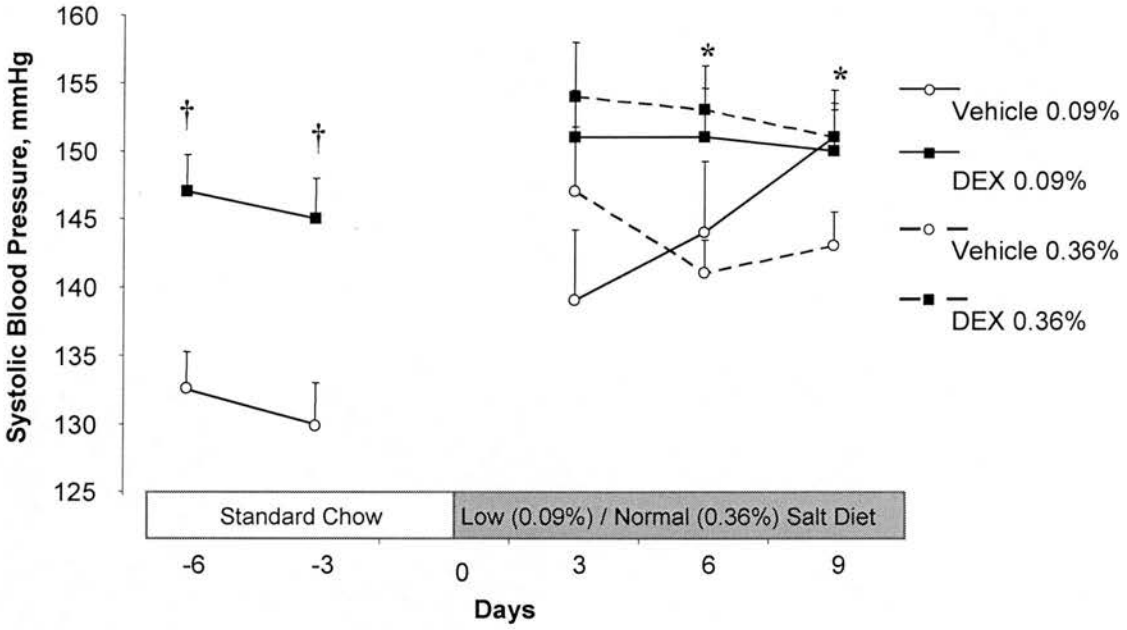
Analyser	Requested Sodium Percentage	Actual Sodium Percentage
Dept. of Clinical Chemistry, Western General Hospital, Edinburgh	**0.3% (Normal)**	**0.1%**
Special Diet Services	**0.3% (Normal)**	**0.085%**
Dept. of Clinical Chemistry, Western General Hospital, Edinburgh	**0.03% (Low)**	**0.36%**
Special Diet Services	**0.03% (Low)**	**0.36%**

5.3.3 Systolic blood pressure before and during manipulation of dietary sodium content

Whilst on standard chow, DEX-treated females displayed significantly elevated systolic blood pressure compared to controls; vehicle 131± 3 mmHg, DEX 147 ± 3 mmHg; repeated measures analysis (prenatal treatment x diet x time), $p = 0.0007$. However, when changed to the new SDS diets containing normal (0.36%) or low (0.09%) sodium levels, blood pressure steadily increased in vehicle-treated animals, particularly those on the low sodium containing diet, so that at the end of the experiment they had systolic blood pressures analogous to DEX-treated offspring {$p = 0.94$, repeated measures analysis (prenatal treatment x diet x time) see **Figure 5-2**}. Conversely, both cohorts of DEX-treated offspring maintained blood pressures similar to those displayed whilst on standard chow (see **Figure 5-2**).

Figure 5-2 **Blood pressure profiles of vehicle and DEX-treated females, prior, and during changes in the sodium content of their diet**

Systolic blood pressure, measured by tail-cuff plethysmography in 6 month old vehicle and DEX-treated offspring on standard chow (n = 12 per group), and subsequently placed on a normal (0.36%; n = 6 per group) or low (0.09%; n = 6 per group) sodium diet for 9 days. All results are mean ± SEM, repeated measures ANOVA (prenatal treatment x diet x time), † $p < 0.001$, * $p < 0.05$ compared with vehicle group.



5.3.4 Effect of dietary sodium on offspring body weight and glucose homeostasis

All offspring continued to gain weight throughout the course of the experiment, with no differences in the gain in body weight noted between offspring cohorts fed either sodium diet (normal sodium diet: vehicle, 8 ± 2g versus DEX, 7 ± 2g; low sodium diet: vehicle 7.5 ± 2g versus DEX, 8 ± 1.5g; 2-way ANOVA (prenatal treatment x diet), p = 0.5).

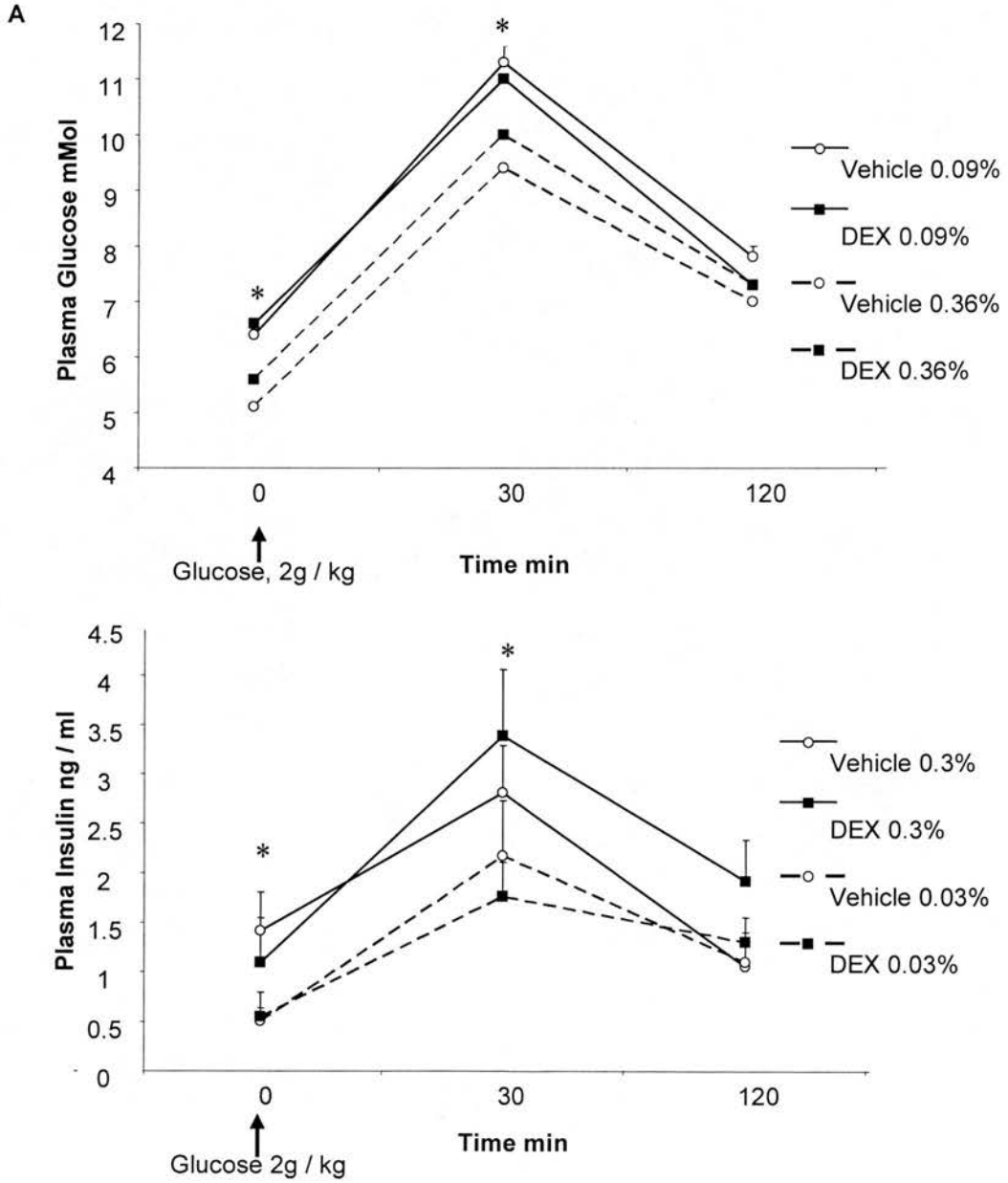
11 days after altering dietary sodium content, offspring underwent an OGTT. Both offspring cohorts fed a low sodium diet had significantly higher basal fasting glucose concentrations compared to those on the normal sodium diet (see **Figure 5-3A**). Furthermore, after an oral glucose load, offspring cohorts fed a low sodium diet had significantly higher 30-min plasma glucose concentrations compared to those fed a normal sodium diet (see **Figure 5-3A**). There were no significant differences at 120 min.

Fasting plasma insulin levels were also significantly elevated in offspring cohorts fed a low sodium diet and at 30 min following an oral glucose load when compared to offspring fed a normal sodium diet (see **Figure 5-3B**). There were no significant differences at 120 min.

Offspring of either prenatal treatment group fed a low sodium diet further showed elevated basal (normal sodium diet, 0.57 ± 0.06 μg/mmol versus low sodium diet, 1.08 ± 0.08 μg/mmol; $p < 0.05$, 2-way ANOVA) and 30 min (normal sodium diet, 1.18 ± 0.09 μg/mmol versus low sodium diet, 1.52 ± 0.07 μg/mmol; 2-way ANOVA (prenatal treatment x diet), $p < 0.05$) insulin-to-glucose ratios when compared to those offspring fed a normal sodium diet. There were no significant differences at 120 min.

Figure 5-3 **Effect of dietary sodium on plasma glucose and insulin responses to an oral glucose load**

Offspring plasma glucose and insulin responses to an oral glucose load. 6 month old female offspring of pregnant rats that received either vehicle or DEX during the last trimester underwent an oral glucose tolerance test 11 days after being switched to a 0.36% (normal) or 0.09% (low) sodium diet. Plasma glucose (**A**) and insulin (**B**) was measured at 0, 30 and 120 min after an oral glucose load. All results are mean ± SEM, 2-way ANOVA (prenatal treatment x diet), n= 6 per group, * $p < 0.05$ compared to low sodium diet

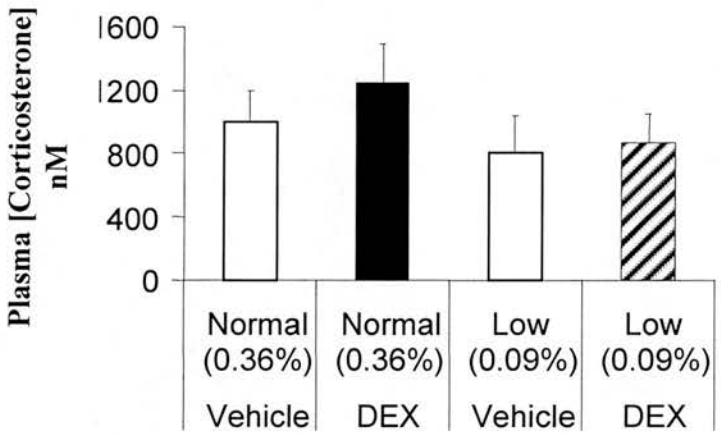


5.3.5 Effect of dietary sodium on offspring plasma corticosterone

As illustrated by **Figure 5-4**, no differences in plasma corticosterone were observed between any of the groups at sacrifice.

Figure 5-4 **Plasma corticosterone levels at sacrifice in female offspring fed normal and low sodium diets**

Plasma corticosterone levels in prenatally vehicle and DEX-treated female offspring, measured in trunk blood taken at sacrifice 14 days post-feeding with either a low or normal sodium diet. All results are mean ± SEM, n= 6 per group.



5.3 Discussion

This chapter relates data accumulated during a course of investigations intended to examine the effects of a 10-fold lower sodium diet on the cardiovascular and metabolic phenotype of the DEX-programmed rat. From the outset, results indicated that it was necessary to re-analyse the diet. The verdict from a local laboratory and Special Diet Services, the company that manufactured the low and normal sodium diets was unanimous; the supposed 'low 0.03% sodium diet' contained 0.36% sodium, whilst the 'normal 0.3% sodium diet' contained 0.09% sodium. Following multiple and prolonged

investigations, it was discovered that the manufacturers had not only incorrectly labeled the supplied diets, but also failed to perform quality control tests on their produce.

Nonetheless, we were still left with diets composed of a normal (0.36%) and low (0.09%) sodium content. In hindsight, this 4-fold difference in sodium content is possibly more clinically and physiologically relevant when compared to the initially proposed 10-fold sodium difference.

Systolic Blood Pressure

In accordance with previous studies (Levitt *et al.* 1996; Benediktsson *et al.* 1993) and sibling offspring (Chapter 4), DEX-treated females displayed higher systolic blood pressures when fed standard rat chow (containing 0.3% sodium).

Vehicle offspring fed either the normal or low sodium diets displayed significantly elevated blood pressure compared to when they were fed normal chow. This was particularly evident in those offspring fed the low sodium diet, whose final blood pressure was identical to that of the DEX-treated offspring. As previously discussed, the hypertension-causing effects of dietary sodium are not unexpected. In terms of mechanism for this hypertension, it is tempting to speculate that it was mediated by dysregulation of the HPA-axis and/ or of the RAS. Indeed, vehicle treated offspring fed either sodium diet displayed hypercorticosteronaemia and hypertension, whereas those fed the low sodium diet also displayed elevated plasma renin activity (PRA) and even higher blood pressure, consistent with this hypothesis.

Interestingly, modifications in dietary sodium did not significantly alter DEX-offspring blood pressure, despite significantly elevated PRA in offspring fed the low sodium diet. This could be explained by the fact that DEX- treated offspring fed a normal sodium diet had analogous PRA and blood pressure values to the vehicle-treated offspring fed the low sodium diet. In Chapter 4, it was demonstrated that an altered RAS (including

increased PRA) mediates in part, the hypertension associated with prenatal DEX treatment. Thus, although dietary sodium alters PRA in DEX-treated offspring, this does not further impact on previously programmed events that cause blood pressure dysregulation. It is tempting to speculate that this is due to the interactions of DEX and a low sodium diet on atrial natriuretic peptide (ANP). ANP, which is increased by DEX treatment (Dananberg *et al.* 1992; Fullerton *et al.* 1991), has been demonstrated to lower BP (by promoting diuresis and natriuresis) even in the face of increased PRA, but only when subjects are fed a low sodium diet (Weidmann *et al.* 1986).

While it may be tempting to explain any blood pressures differences as a technical artifact of measurement (see Chapters 2 and 6 for in-depth discussion); tail-cuff plethysmography was sufficiently sensitive in detecting blood pressure differences when the offspring were fed standard chow. Moreover, when rodents fed a low sodium diet during weaning became hypertensive, measurements made in the tail correlated well with measurements made simultaneously by an intra-arterial catheter (Webb *et al.* 1987). Of course, fastidious blood pressure profiling could only be achieved by radio-telemetry (see Chapters 2 and 6 for further comment).

Plasma renin activity and plasma aldosterone and corticosterone levels

In the present study low dietary sodium produced a significant increase in PRA in both offspring, whilst the trend for plasma aldosterone to be higher did not reach significance. As the supplied low sodium diet contained three times the desired low sodium concentration, it is possible that whilst sufficiently low to increase PRA, our diet was inadequate to stimulate a considerable increase in plasma aldosterone levels. Alternatively, it could be that the assay (Cartledge and Lawson 2000), and indeed the physiology of aldosterone are more variable. For example, the dietary and plasma levels of potassium (a key stimulator of aldosterone secretion) and plasma ANP (known to suppress aldosterone, even in the face of increased PRA, Weidmann *et al.* 1986) in the current study are unknown. In one study (Malee & Mellon 1991), rats required a totally

sodium deficient diet before alterations in aldosterone synthase were observed (although plasma aldosterone levels were not examined). On the other hand, rodents fed a low sodium diet during weaning displayed modified PRA and hypertension, without alterations in plasma aldosterone (Webb *et al.* 1987), thereby mirroring our observations. Moreover, our findings are in direct agreement with the 'low sodium diet' model of prenatal programming (Battista *et al.* 2002), whose offspring also display hypercorticosteronaemia and hypertension. Clearly, further investigations in our model are warranted before we can reliably report on the nature of sodium-induced RAS changes.

Elevated offspring corticosterone levels could be associated with hypertension as previously discussed, and are possibly due to the well-documented stress associated with dietary change (Hatton *et al.* 1987; Brindley *et al.* 1985).

Glucose homeostasis

Interestingly, both vehicle and DEX-treated offspring, fed a low sodium diet for 11 days became glucose intolerant and insulin resistant, when compared to offspring fed a high sodium diet. How could a low sodium diet 'overwrite' programming and render both vehicle and DEX-treated offspring insulin resistant? Decreased insulin clearance as a result of RAS activation (observed in both cohorts fed a low sodium diet) may be responsible for this phenomenon (Lender *et al.* 1997). Indeed, decreased blood flow to the liver as a result of Ang II (Ichikawa *et al.* 1979) can diminish insulin clearance (Meland *et al.* 1994), and has been proposed to underlie the insulin resistance associated with low salt diet feeding in wistar rats (Prada *et al.* 2000). Thus, it is tempting to speculate that the increased basal plasma insulin levels observed in this study are related to high plasma Ang II concentration. The association between a low sodium diet and insulin resistance also concurs with human studies of both healthy volunteers and patients with essential hypertension. (Fliser *et al.* 1995; Meland *et al.* 1997). Additionally, insulin has been postulated to play a role in the pathophysiology of

essential hypertension (see section 1.4.2e), and could therefore possibly contribute to hypertension in this study.

Summary

This study reveals that a 4-fold reduction in dietary sodium results in hypertension in prenatally vehicle treated animals, and supports a role for both the HPA and RAS in mediating this. Conversely, whilst a lower sodium diet intensifies RAS dysregulation in DEX-treated offspring, this does not exacerbate their programmed blood pressure phenotype. Acute exposure to a lower sodium diet is sufficient to cause glucose intolerance and insulin resistance in adult rats, irrespective of their prenatal treatment. Repetition of many of these measurements under the initially proposed dietary conditions, together with the addition of plasma AngII and ANP measurements and radiotelemetric blood pressure monitoring, would be instructive in further elucidating the phenotype of the DEX-programmed rat.

Incidentally

As a result of legal action arising from this study, Special Diet Services now ensures independent testing of every bespoke diet, thereby sparing other researchers the agonising experience of wasting time, animals and funds.

Altered sympathetic responses mediate the stress-induced hypertension associated with prenatal dexamethasone in the rat

6.1 Introduction

In recent years, there has been growing evidence for an effect of intrauterine development on disease risk in later life (Law *et al.* 2001; Curhan *et al.* 1996a; Curhan *et al.* 1996b; Fall *et al.* 1998; Osmond *et al.* 1993; McCance *et al.* 1994; Barker *et al.* 1990; Law *et al.* 1993; Yarbrough *et al.* 1998). Global epidemiological studies indicate that low birth weight, a surrogate measure of an adverse intrauterine environment, is associated with an increased risk of type 2 diabetes (McCance *et al.* 1994), hypertension (Barker *et al.* 1990) and cardiovascular disease in adulthood (Barker 1999; Curhan *et al.* 1996b). To explain these findings, the physiological concept of prenatal 'programming' has been developed, whereby a factor acting during critical periods of fetal life alters the development of organs, resulting in permanently altered function and eventually disease (Barker *et al.* 1993; Edwards *et al.* 1993).Whilst the pathophysiological mechanisms underlying this relationship between low birth weight and subsequent adulthood disease remain to be fully established, both fetal malnutrition (Barker *et al.* 1993) and *in utero* overexposure to glucocorticoids (Benediktsson *et al.* 1993) have been proposed as mediators of permanent programming of cardiovascular and metabolic phenotypes.

Fetal 'programming' may be investigated in a more controlled manner by using laboratory animals that age rapidly and have similar genetic backgrounds. We and others have long demonstrated in rats that prenatal exposure to the synthetic glucocorticoid dexamethasone (DEX), commonly used in obstetric practice, produces permanently impaired glucose intolerance, insulin resistance and hypertension in the adult offspring (Levitt *et al.* 1996b; Nyirenda *et al.* 1998; Nyirenda *et al.* 2001; Langdown *et al.* 2001; Sugden *et al.* 2001). Similarly, overexposure of endogenous maternal glucocorticoids by inhibition of the feto-placental 'barrier enzyme', 11beta hydroxysteroid dehydrogenase

type 2, programmes the same adulthood cardiovascular and metabolic pathologies (Welberg *et al.* 2000; Lindsay *et al.* 1996a; Lindsay *et al.* 1996b). Although programmed increases in the expression of key hepatic gluconeogenic enzymes may underpin later hyperglycaemia (Nyirenda *et al.* 1998; Nyirenda *et al.* 2001), little is known of the mechanisms by which glucocorticoids programme hypertension.

In rats, prenatal DEX, specifically in the last trimester, affects the development and maturation of specific organs related to blood pressure control and maintenance; notably the developing heart, kidney, vasculature and brain. Alterations in the activity or responsivity of the sympathetic nervous system (SNS), either systemically or in one of these specific regions, could provide one potential explanation for the manner in which prenatal glucocorticoids promote adulthood hypertension. Indeed, in humans, increased SNS activity has been proposed as a mechanism to explain the pathogenesis of glucocorticoid-induced hypertension (Whitworth 1994; Whitworth *et al.* 1995; Saruta 1996). Consistent with this hypothesis, elevated SNS activity, established *in utero*, has been demonstrated as one mechanism to link small size at birth with raised blood pressure in adult life (Phillips & Barker 1997). In rats, prenatal DEX is known to interfere with the development of cardiac, and central sympathetic innervation and activity (Bian *et al.* 1993b; Slotkin *et al.* 1992), and to permanently alter the pattern and expression of alpha and beta adrenergic receptors (Huff *et al.* 1991; Bian *et al.* 1992); thereby possibly altering vascular responsivity to vasoconstrictors. Similar findings have been reported with DEX administration during pregnancy in sheep, and are proposed to mediate the increased cardiovascular responses in glucocorticoid treated fetuses (Tseng *et al.* 1995; Padbury *et al.* 1995; Stein *et al.* 1994; Stein *et al.* 1993).

In the current study, we hypothesized that prenatal DEX-administration in the final week of gestation increases blood pressure in the adult offspring by altering sympathetic nerve responsivity; to investigate this, we used the method of radiotelemetry (Anderson *et al.* 1999), which enables the remote recording of blood pressure in conscious, unrestrained offspring. The pressor response of the mesenteric vasculature to noradrenaline, vasopressin and potassium chloride, in both male and female offspring were also assessed.

6.2 Methods

6.2.1 Animals

Female rats and their litters were maintained, bred, and administered with vehicle or DEX during their third week of pregnancy as described in Chapter 2. The following represent a new cohort of prenatally treated animals and are not the same as those described in Chapter 4. Male and female offspring were weighed at 3, 7, 14 and 21 days of age, and at three weekly intervals thereafter throughout postnatal life.

6.2.2 Radiotelemetric blood pressure measurement

Blood pressure (systolic, diastolic, and mean pressure), heart rate, and activity were assessed by radiotelemetry in randomly selected littermates (5 male and 5 female, from 8 vehicle and 8 DEX litters) at 7-8 months of age, as outlined in Chapter 2.

6.2.3 Induction of stress by disturbance, weighing, and restraint procedure

To assess responses to stress, offspring were subjected to a series of graded stressors from simple disturbance (a researcher entering their room), to being weighed, and placed in a Perspex cylinder for 15 min. To evaluate the stress response to weighing, haemodynamic measurements were recorded prior to removal of the rat from its cage, and again immediately and at 5 min post-weighing, when the animal was replaced in its home cage. For the restraint procedure, haemodynamic measurements were collected prior to the rat being placed in the cylinder, throughout the 15 min restraint period, and at 15 min post-restraint, when the animal was returned to its cage. A rest period of two days was allowed between experiments.

6.2.4 Haemodynamic responses to alterations in catecholaminergic mechanisms

Haemodynamic responses were analysed 2 hr following the depletion of catecholamines by i.p. reserpine ($0.5mg.kg^{-1}$), and during a 15 min restraint test, performed 4 hr post-

reserpine administration. After a 1-week recovery period, haemodynamic responses were collected for 15 min following a low dose of i.p. d-amphetamine ($0.5mg.kg^{-1}$), aimed to cause systemic catecholamine release.

6.2.5 Vascular responses to noradrenaline, vasopressin, and potassium chloride

12 pairs of mesenteric vasculature preparations from both male and female offspring aged between 4-4.5 months were simultaneously tested with increasing concentrations of agonists in the following order: NA (0.1- 20μM; Sigma-Aldrich, UK), AVP (0.5- 20 nM; Sigma-Aldrich, UK) and KCl (25-125mM), as described in Chapter 2.

6.2.6 Statistical analyses

All data are expressed as mean ± SEM.

Radiotelemetry data were compared using two-way or multiple ANOVA followed by a LSD post-hoc multiple comparisons test where appropriate. Values were considered significant when $p < 0.05$.

For each mesenteric preparation, the maximal response and the concentration of agonist required for a 50% response (EC_{50}) were determined. Differences in the mean log EC_{50} and maximal response values between vehicle and DEX-treated offspring mesenteric vasculatures were compared using Student's unpaired t-test. Data were further analysed by 2-way ANOVA (prenatal treatment x dose of agonist) followed by a LSD post-hoc multiple comparisons; results were considered significant when $p < 0.05$.

6.2 Results

6.3.1 Gestational weight gain, birth phenotype and catch-up growth

As outlined in **Table 6-1**, DEX administration throughout the final week of gestation again resulted in a significant reduction in maternal weight gain, with no differences noted in the length of gestation, litter size, ratio of male to female pups born or pup viability. DEX treatment caused a significant reduction in the birth weight of offspring, which was observed to the same degree in both male and female pups, consistent with previous studies.

By weaning (postnatal day 21) and throughout adult life, the gain in body weight of male and female offspring, treated with vehicle or DEX were similar, *i.e.* full catch-up growth had occurred (data not shown).

Table 6-1 Comparison of gestational and birth parameters in vehicle and DEX treated cohorts

Maternal weight before treatment, maternal weight gain during the last week of pregnancy (days 15-21), gestation length, litter size, male: female ratio, and group, male, and female offspring birth weight. Results are mean ± S.E.M, Student's t-test, * $p < 0.05$, † $p < 0.01$ compared with vehicle group.

	Maternal weight before treatment	Maternal weight gain	Gestation length	Litter size	Male: female ratio	Birth weight		
						Group	Male	Female
	(g)	(g)	(d)				(g)	
Vehicle	365 ± 14 (n = 8)	97 ± 5	22 ± 0	13 ± 2.0	1.1 ± 0.1	6.6 ± 0.01 (n = 99)	6.6 ± 0.09 (n = 55)	6.5 ± 0.08 (n = 44)
DEX	363 ± 6 (n = 9)	58 ± 5*	22 ± 0	12 ± 1.8	1.2 ± 0.2	5.7 ± 0.01† (n = 108)	5.6 ± 0.08† (n = 56)	5.7 ± 0.07† (n = 52)

6.3.2 Radiotelemetry monitoring of offspring blood pressure, heart rate, and activity under basal conditions

Circadian variations of blood pressure, heart rate, and activity were detected in all offspring of the prenatal treatment groups; being highest early in the morning and falling to their lowest during the afternoon (see **Table 6-2** and **Figure 6-1A**). Both DEX-treated male and female offspring had lower active (dark) phase systolic blood pressure when compared to controls (see **Table 6-2**). Mean arterial blood pressure was also significantly lower in DEX-treated offspring (see **Figure 6-1A**). There were no significant differences between control and DEX-treated offspring in diastolic blood pressure (see **Table 6-2**), heart rate (see **Table 6-2** and **Figure 6-1B**) or activity (see **Table 6-2**).

Table 6-2 Offspring baseline cardiovascular and activity phenotypes

Cardiovascular parameters and activity measured under basal conditions in adult offspring of vehicle and DEX-treated dams. Results are mean ± S.E.M, 2-way ANOVA (prenatal treatment x gender), * $p < 0.05$, compared with vehicle group.

Parameter	**Vehicle ♂** (n = 5)	**Dex ♂** (n = 5)	**Vehicle ♀** (n= 4)	**Dex ♀** (n = 5)
Systolic BP, mmHg				
Night	153 ± 7	143 ± 10*	144 ± 6	135 ± 10*
Day	137 ± 7	134 ± 10	127 ± 5	125 ± 9
Diastolic BP, mmHg				
Night	111 ± 6	105 ± 10	106 ± 5	105 ± 11
Day	102 ± 5	98 ± 8	97 ± 5	95 ± 9
Heart Rate, bpm				
Night	379 ± 13	378 ± 37	414 ± 17	410 ± 24
Day	298 ± 19	319 ± 41	310 ± 12	323 ± 22
Activity				
Night	4 ± 1	4 ± 1	9 ± 3	6 ± 3
Day	1 ± 0.4	1 ± 0.5	1.4 ± 0.1	1.4 ± 1

Figure 6-1A Offspring basal mean arterial blood pressure patterns

Basal mean arterial blood pressure (MAP) in male and female offspring exposed to vehicle (---) or DEX (—) during the last week of gestation. Data are expressed as 6-hour rolling averages over 3 days. Values are mean ± S.E.M, 2-way ANOVA (prenatal treatment x gender), $n = 4$ -5 of each sex per group, * $p < 0.05$ compared with vehicle group at midnight and midday.

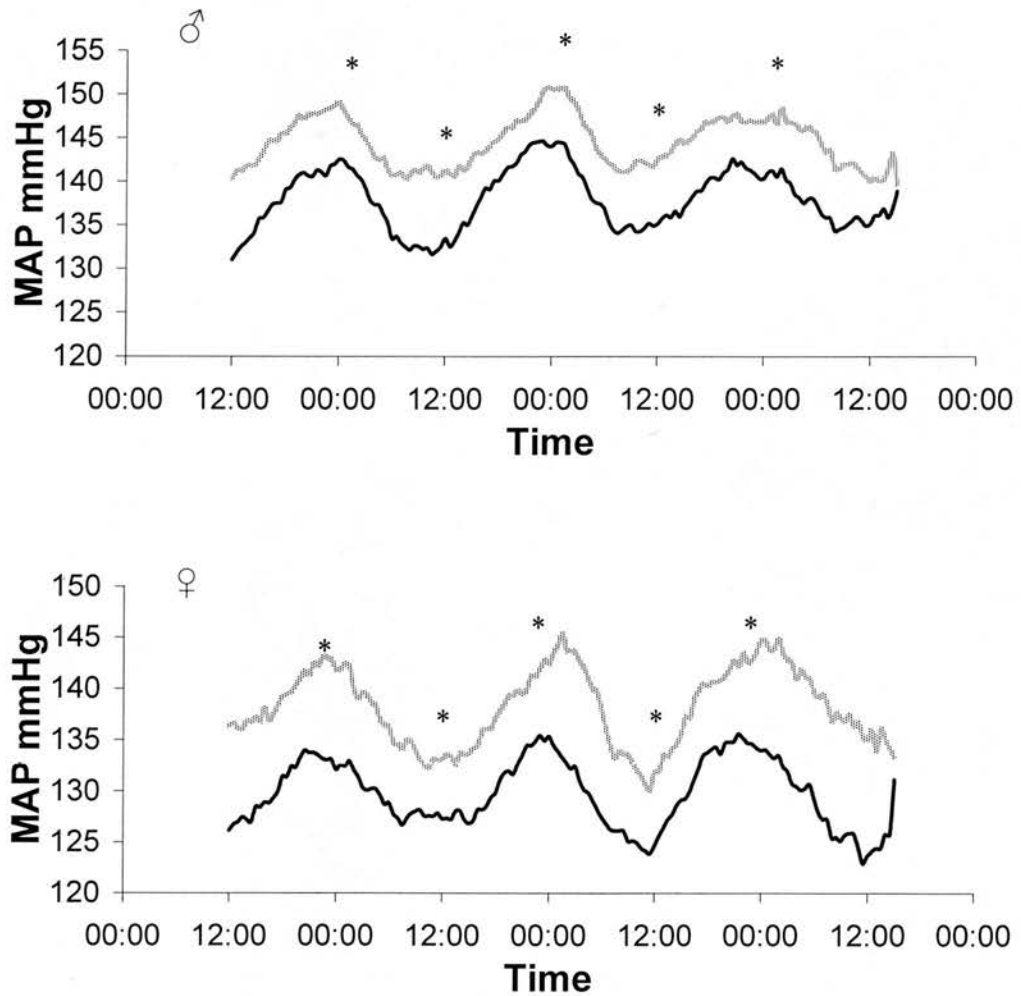
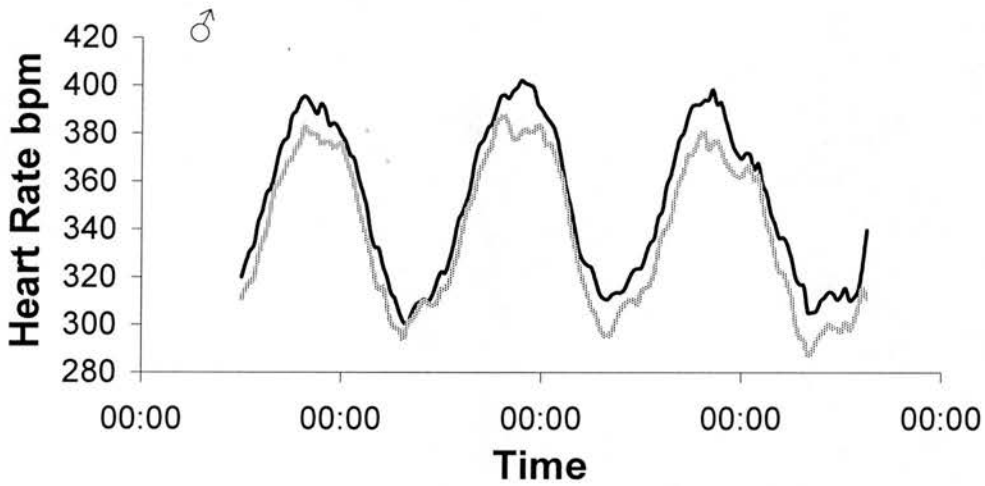
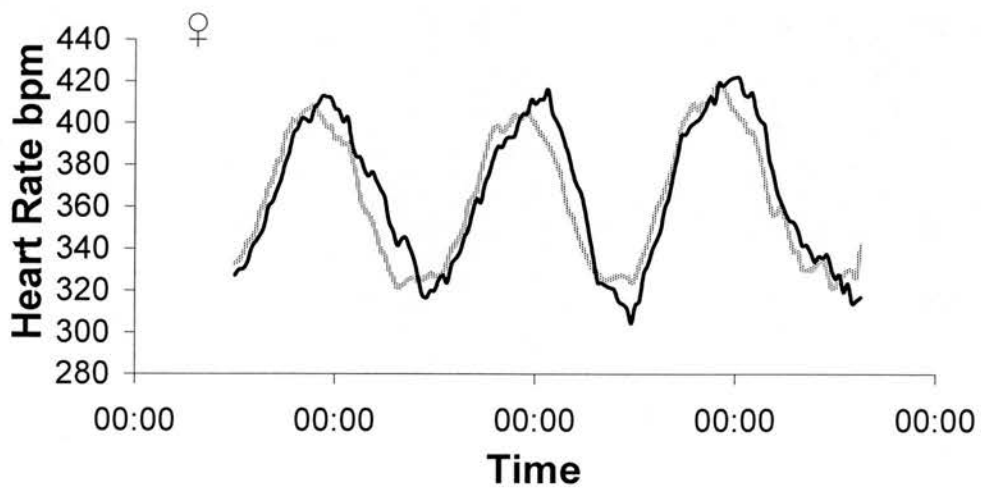


Figure 6-1B Offspring basal heart rate patterns

Basal heart rate patterns in male and female offspring exposed to vehicle (---) or DEX (—) during the last week of gestation. Data are expressed as 6-hour rolling averages over 3 days. Values are mean ± S.E.M, 2-way ANOVA (prenatal treatment x gender), compared with vehicle group at midnight and midday, $n = 4$ -5 of each sex per group.





6.3.3 Offspring haemodynamic and activity responses to graded stressors

In utero exposure to DEX has been shown to cause a lifelong elevation in basal blood pressure when measured by tail-cuff plethysmography and carotid cannulation. An exaggerated stress response to these measurement techniques would account for this hypertension. Therefore, we examined whether prenatal exposure to DEX was associated with a hypertensive response to stress. As shown in **Figure 6-2A**, DEX-treated offspring are exquisitely hypersensitive to stress, displaying graded hypertensive responses when disturbed by a researcher entering their room, and when restrained. Even weighing elicits a hypertensive response in DEX treated offspring (change in systolic blood pressure, vehicle 10 ± 2 mmHg, DEX 19 ± 3 mmHg; $p < 0.05$). Moreover, DEX-treated offspring maintain their hypertension for longer once released from restraint (see **Figure 6-2A**), and after weighing (change in systolic blood pressure 5 min post-weighing, vehicle, 7 ± 5 mmHg, DEX 23 ± 5 mmHg ; $p < 0.05$). Dex-treated offspring further displayed significantly increased changes in heart rate in response to being weighed (vehicle 117 ± 17 bpm, DEX 156 ± 25 bpm; $p < 0.05$), and held in restraint (vehicle 86 ± 11 bpm, DEX 136 ± 19 bpm; $p < 0.05$), which were not associated with changes in activity (data not shown).

6.3.4 Offspring responses to alterations in catecholaminergic pathways

To assess the role of the sympathetic nervous system in mediating this stress induced hypertension, we depleted nerve terminal catecholamines, using a low dose of reserpine. DEX-treated offspring displayed a greater fall in mean arterial blood pressure and heart rate (Table 3). Moreover, their previously demonstrated hypertensive responses pre-, (vehicle -16 ± 3 mmHg, DEX 22 ± 4 mmHg; $p = 0.1$), during (vehicle 2 ± 5 mmHg, DEX 1 ± 3 mmHg; $p = 0.6$) and post-restraint (vehicle -5 ± 2 mmHg, DEX -4 ± 3 mmHg; $p = 0.86$) were completely abolished (see **Figure 6-2B**). Conversely, a low dose of d-amphetamine, to induce catecholamine release, resulted in a significantly greater increase in DEX-treated offspring's mean arterial blood pressure and heart rate (see **Table 6-3**). No significant differences in activity were noted between offspring's response to either reserpine or d-amphetamine (see **Table 6-3**).

Figure 6-2 Offspring blood pressure response to stress and inhibition of catecholamine synthesis

A. Stress-induced mean arterial blood pressure changes in prenatal vehicle and DEX-treated offspring, following disturbance, restraint, and at 15 min post- restraint. **B.** Inhibiting catecholamine synthesis (reserpine; 0.5mg/kg) abolishes the exaggerated stress-induced elevations in mean arterial blood pressure, previously displayed by prenatal DEX-treated offspring during and post restraint. Values are mean ± S.E.M, 2-way ANOVA (prenatal treatment x gender), n = 4-5 of each sex per group in **A**, and 3-4 of each sex per group in **B**, * $p < 0.05$ compared with vehicle group.

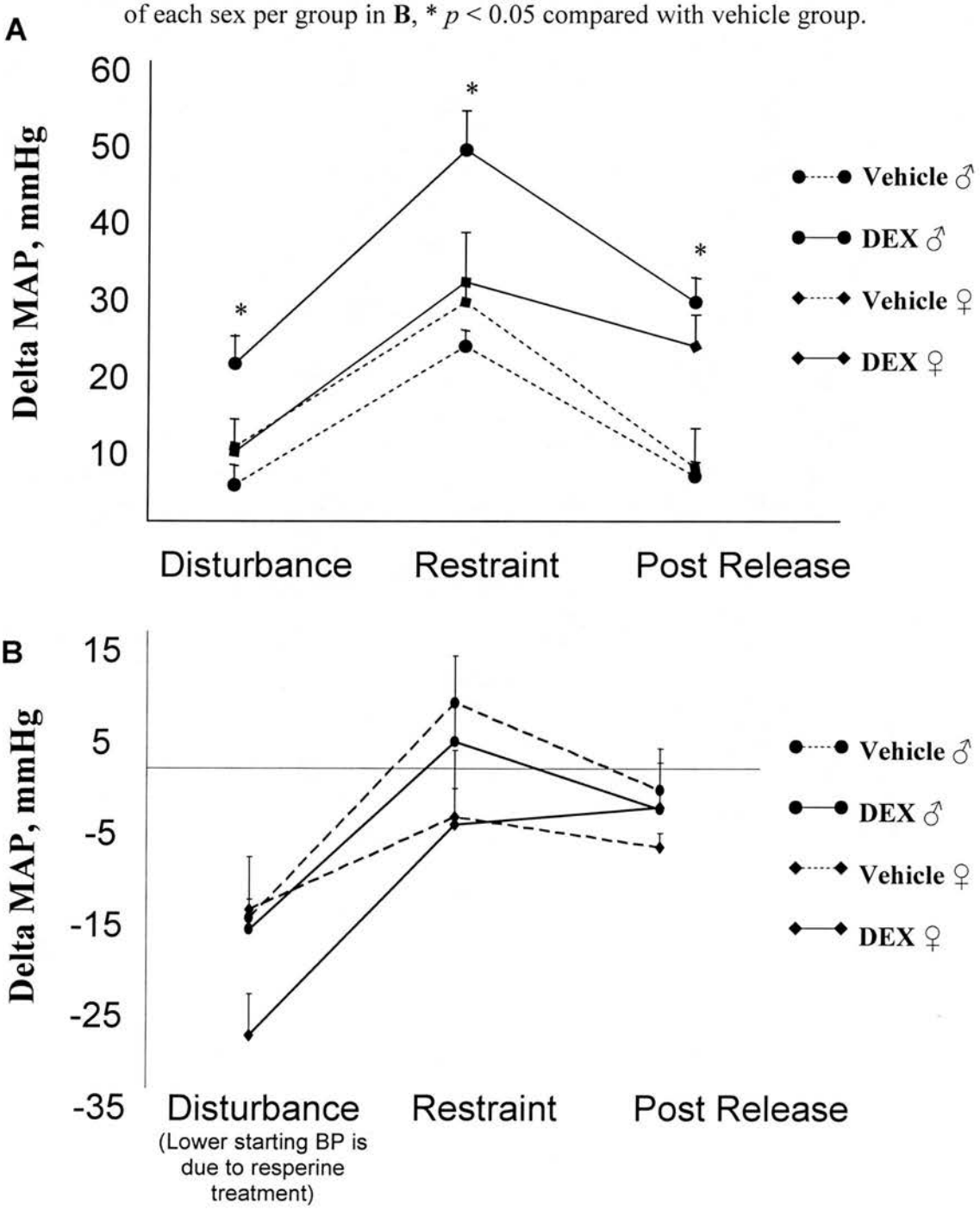


Table 6-3 Effect of reserpine and d-amphetamine on offspring haemodynamic and activity patterns

Alterations in baseline haemodynamic parameters and activity post reserpine (catecholamine synthesis inhibition) and d-amphetamine (catecholamine release) administration, in adult offspring of vehicle and DEX-treated dams. Results are mean ± S.E.M, 2-way ANOVA (prenatal treatment x gender), * $p < 0.05$, compared with vehicle group.

	Vehicle Offspring (n = 7; 4 ♂, 3 ♀)	**Dex Offspring** (n = 7; 4 ♂, 3 ♀)
Reserpine (0.5mg/kg; i.p.)		
Delta MAP, mmHg	-16 ± 2	-24 ± 3*
Delta Heart Rate, bpm	-74 ±16	-145 ± 18*
Delta Activity	-3 ± 1.3	-2.5 ± 1.2
D-amphetamine (0.5mg/kg; i.p.)		
Delta MAP, mmHg	14 ± 0.5	26 ± 3*
Delta Heart Rate, bpm	32 ± 3	67 ± 11*
Delta Activity	13 ± 2	13 ± 3

6.3.5 Responsiveness of offspring mesenteric vasculature to noradrenaline, vasopressin, and potassium chloride

Male Offspring

The responses of the mesenteric vasculature to the three agonists are shown in **Figures 6-3A**, **B**, and **C**. Basal perfusion pressures were similar in the two groups of rats. Infusion of all agonists caused a concentration-dependent increase in perfusion pressures, with plateaux reached in both groups at 5μM for noradrenaline, 10nM for vasopressin, and 125mM for potassium chloride. However, tissues from DEX-treated offspring were significantly more responsive; the maximal increments were greater to each agonist tested, and the EC_{50} values were lower for both noradrenaline and vasopressin (see **Table 6-4**).

Table 6-4 Mesenteric vascular function in vehicle and DEX-treated male offspring

Maximal contraction and responsiveness to vascular agonists (as assessed by EC_{50}), in the mesenteric vasculature from vehicle and DEX-treated male offspring. Values are mean ± S.E.M; n= 6 per group; Student's t-test,* $p < 0.05$, † $p < 0.01$, compared with vehicle group

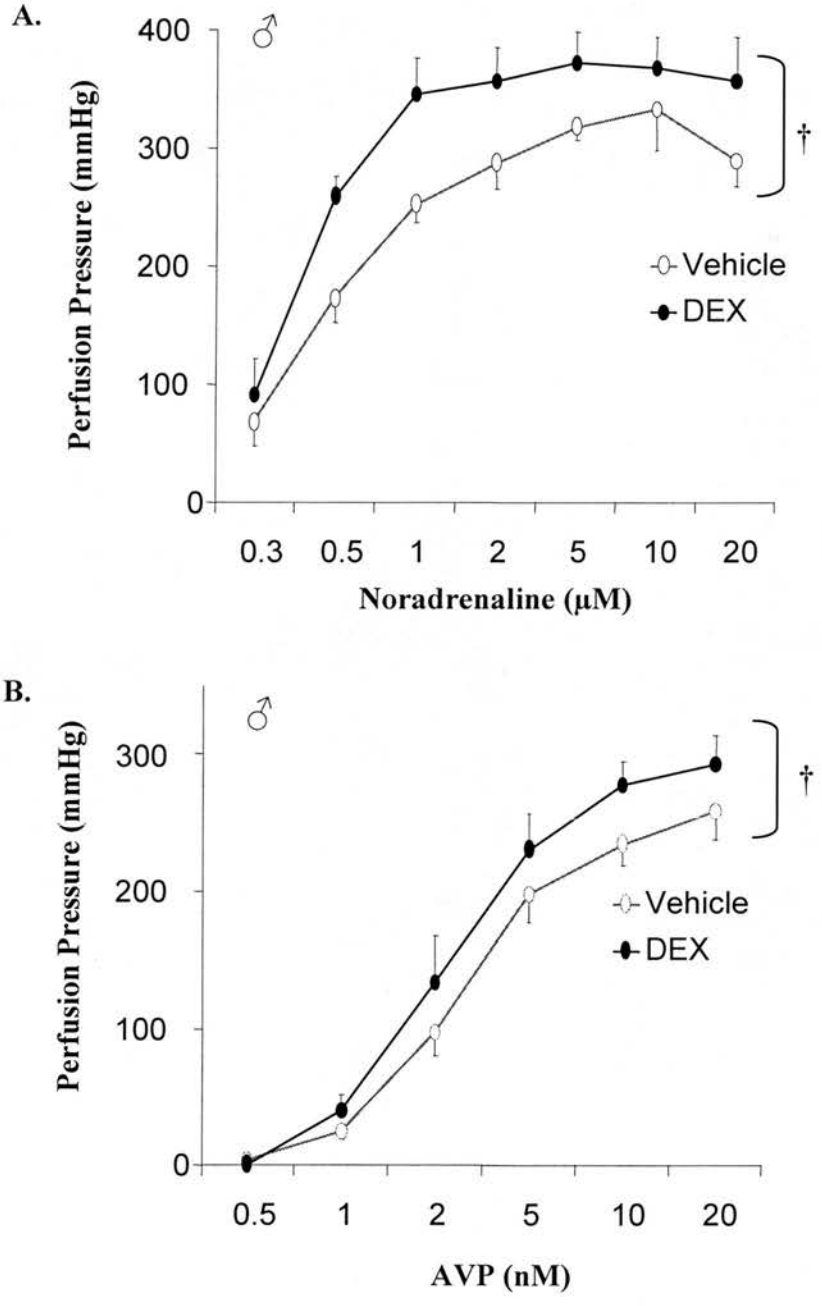
Vascular parameter	Agonist	Vehicle	DEX
Maximal Contraction (mmHg)	Noradrenaline	318 ± 12	371 ± 25†
	Vasopressin	260 ± 19	298 ± 19*
	KCl	270 ± 27	327 ± 16*
EC_{50}	Noradrenaline (μM)	0.5 ± 0.06	0.4 ± 0.02†
	Vasopressin (nM)	3.0 ± 0.20	2.5 ± 0.30*
	KCl (mM)	63.0 ± 5	67 ± 3 (p = 0.27)

Female Offspring

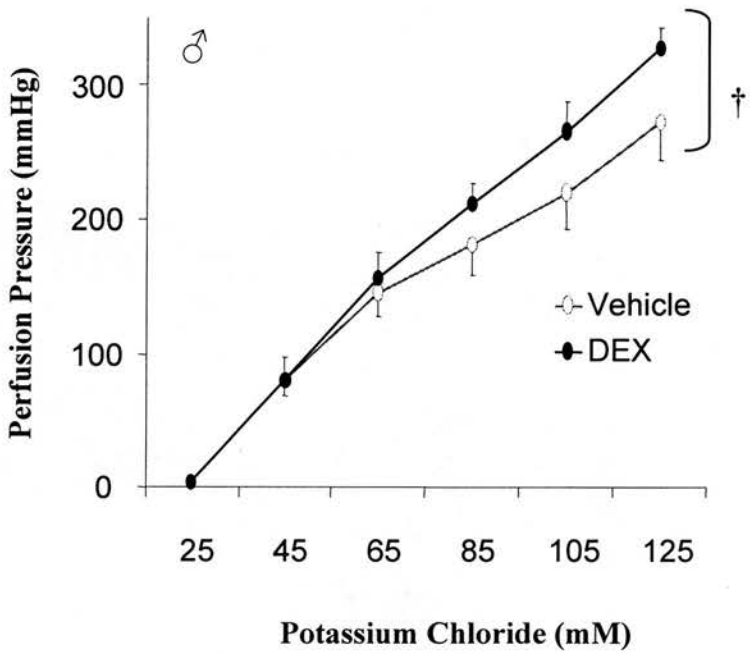
Similar basal perfusion pressures were noted in the two groups. Noradrenaline caused a concentration-dependent increase in perfusion pressures, with plateaus reached at 2μM in both treatment groups (see **Figure 6-3D**). However, the mesenteric vasculatures from DEX-treated offspring were significantly more responsive to noradrenaline, (as analysed by 2-way ANOVA), even though the maximal contraction (Vehicle, 344 ± 40mmHg versus DEX, 373 ± 30; $p = 0.16$) and EC_{50} (Vehicle, 0.43 ± 0.02μM versus DEX, 0.39 ± 0.03μM; $p = 0.16$) values were similar for both treatment groups. DEX treatment did not affect the pattern of responses to either vasopressin or potassium chloride. Maximal responses in both groups were achieved at 5nM vasopressin, and 125mM potassium chloride (data not shown).

Figure 6-3 Constrictor responses of mesenteric vasculature from adult male and female rats treated with vehicle or DEX *in utero*

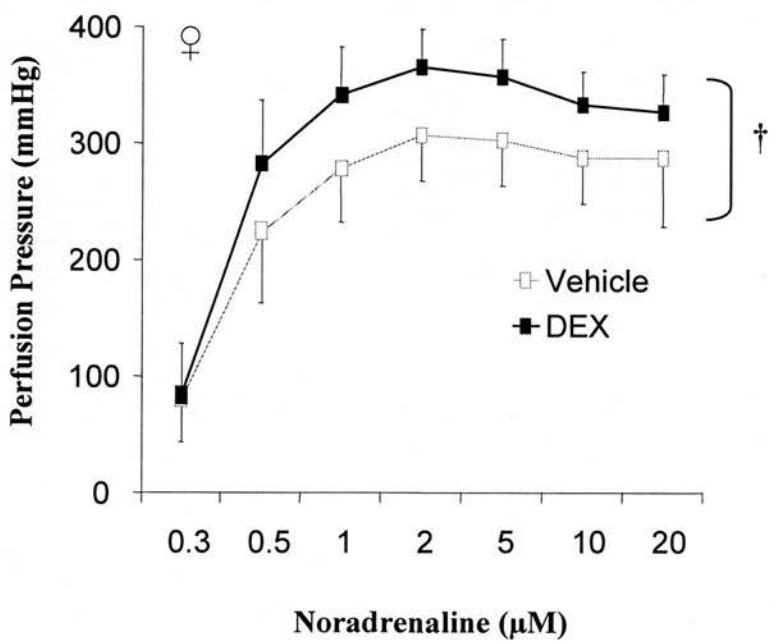
Male offspring dose-response curves to noradrenaline (**A**), vasopressin (**B**) and potassium chloride (**C**); and female offspring dose-response curve to noradrenaline (**D**), in the isolated mesenteric vasculature from vehicle and DEX-treated rats. Results are mean ± S.E.M, $n = 6$ per group, 2-way ANOVA (prenatal treatment x dose), † $p < 0.01$ compared with vehicle group.



C.


♂
Perfusion Pressure (mmHg)
300
200
100
0
25
45
65
85
105
125
Potassium Chloride (mM)
Vehicle
DEX
†

D.


♀
Perfusion Pressure (mmHg)
400
300
200
100
0
0.3
0.5
1
2
5
10
20
Noradrenaline (μM)
Vehicle
DEX
†

6.4 Discussion

The novelty of this study lies in the observation that prenatal DEX during the last week of pregnancy actually results in ***lower*** basal blood pressure in the adult offspring; with the hypertensive phenotype only being noted when these offspring are subjected to a stressor. Moreover, DEX-treated offspring maintain this stress-induced hypertension for longer following removal of the stressor. These hypertensive responses are mediated by alterations in the responsivity of the SNS, being ameliorated by the inhibition of catecholamine synthesis, and further exaggerated by the promotion of systemic catecholamine release. Additionally, DEX-treated offspring display greater sensitivity to a variety of vasoconstrictors in the isolated mesenteric vasculature.

Radiotelemetry, as a method of blood pressure measurement in rats has myriad advantages over both tail-cuff plethysmography (TCP) and carotid cannulation (CC), as it enables the recording of systolic and diastolic pressures in conscious, unrestrained, and unanaesthetized animals. The majority of reports in which offspring blood pressure has been assessed in rat models of fetal programming have relied on TCP (Langley-Evans 1997; Woodall *et al.* 1996; Langley & Jackson 1994) or CC (Levitt *et al.* 1996a; Benediktsson *et al.* 1993). TCP, which only records systolic blood pressure, not only involves preheating of the animal prior to measurement, but also requires considerable restraint, as does CC. Naturally, both preheating and restraint could artifactually cause a substantive elevation in basal blood pressure while failing to observe a selective increase in pressor responses (Tonkiss *et al.* 1998). Furthermore, radiotelemetry also provides a contrast between day (sleep) and the physiologically more accurate night (awake) values, together with heart rate and an estimate of activity. Thus, serious stress effects, together with the day-time recording of blood pressure, would account for the previously demonstrated hypertension associated with prenatal glucocorticoid programming, when assessed by TCP or CC. The observation in the current study that DEX-treated offspring had reduced systolic and mean arterial blood pressure was completely unexpected.

To the best of our knowledge, this is the first investigation to have undertaken a comprehensive longitudinal study with the use of radiotelemetry for the measurement of blood pressure in a prenatal glucocorticoid model of fetal programming. In rats, only two previous dietary models of fetal programming (Tonkiss *et al.* 1998; Khan *et al.* 2003) and one of intrauterine growth restriction have utilised radiotelemetry (Jansson & Lambert 1999). In one, blood pressure was measured over a single 24-hour time period, and uncovered a small (4mmHg) significant rise in the awake-phase diastolic blood pressure in the male offspring of dams fed a diet with significantly reduced protein content (by 19%) (Tonkiss *et al.* 1998). Female offspring were not studied. Interestingly, in that study the offspring of the malnourished rats displayed a greater hypertensive response to stress (ammonia odour) when compared to controls, thereby not only highlighting the importance of recording in conscious unrestrained animals, but also hinting at a potential cause of programmable hypertension.

The impetus for examining pressor responses to stress stemmed not only from this evidence, and the possible explanation for the hypertension uncovered by TCP or CC, but also from the simple observation in the current study that merely entering the room in which the offspring were housed was sufficient to drive hypertension selectively in the DEX-treated offspring. When further subjected to a moderate stressor (weighing) and the more severe restraint stress, similar to that used in TCP, the pressor responses of the male and female DEX-treated offspring were significantly augmented, suggesting increased sensitivity to environmental stress. An increased reactivity to stressful environmental stimuli may lead to established hypertension through frequent transient blood pressure elevations (Folkow & Hallbäck 1978), and whether these offspring would eventually become permanently hypertensive warrants further study. Indeed, the DEX-treated offspring maintained their hypertension longer after the removal of each stressor.

Insight into the potential mechanisms of this stress-induced hypertension may be gained from the observation that DEX-treated offspring had higher heart rates (i.e. a marker, although indirect, of cardiovascular adrenergic drive) associated with each of their

hypertensive stress responses, indicating increased sympathetic nerve activity and/or responsivity. Depletion of nerve terminal catecholamine stores with reserpine (an Uptake-1 inhibitor) was sufficient to abolish the effect of DEX on the hypertensive response and accompanying tachycardia pre- and post-restraint stress, whilst the same dose of d-amphetamine led to a greater increase in these basal haemodynamic parameters, further strengthening the evidence for a more responsive SNS. The SNS participates in several stress responses, even in fetal life (McMillen *et al.* 2001), and therefore, prenatal exposure to various stressors might affect the development of sympathetic innervation or its regulation. For example, in rats, the offspring of prenatal DEX-treated dams have increased noradrenaline turnover (a measure of neuronal impulse) in central brain regions concerned with blood pressure control (Slotkin *et al.* 1992), whilst cardiac noradrenaline levels and turnover are suppressed (Bian *et al.* 1993a). In line with these observations, continuous gestational exposure to nicotine impaired the maturation of both central and peripheral catecholaminergic pathways in rats (Navarro *et al.* 1988), and a study on the offspring of rats kept in hypoxia during pregnancy showed altered development of sympathetic centers involved in blood pressure regulation (Peyronnet *et al.* 2002). Most interestingly, in the latter study, the adverse effects on blood pressure regulation persisted throughout adulthood. Similarly, ligation of the umbilical artery, an experimental model of fetal malnutrition, resulted in alterations in SNS activity in both the newborn sheep (Oyama *et al.* 1992) and adult female rat offspring (Jansson & Lambert 1999). Taken together, these animal data firmly indicate that adverse influences *in utero* are capable of impairing the development and function of the SNS. A recent study in low birth weight adults proposed that their lower resting muscle sympathetic nerve activities were a function of altered SNS development (Weitz *et al.* 2003). It is tempting to speculate that the demonstrated augmented SNS responsivity observed in our DEX-treated offspring is a function of altered regulation of adrenergic receptor density and coupling to or functional activity of signal transduction mechanisms, though this remains to be elucidated.

Increases in peripheral vascular resistance and cardiac output may also contribute to the stress-induced hypertension observed in our prenatal DEX-treated offspring and have been postulated to mediate the increases in blood pressure during glucocorticoid treatment both pre- and post-natally (Derks *et al.* 1997). Studies using synthetic glucocorticoids, such as DEX or bethasmethasone, suggest that the increased blood pressure is independent of mineralocorticoid-mediated effects, such as sodium retention or volume expansion (Grunfeld 1990). Rather, it appears that glucocorticoids directly alter vascular responsiveness to circulating vasoactive agents. In the present study, the pattern of dose-response curves in the mesenteric vasculature of DEX-treated offspring showed greater *in vitro* pressor responses to noradrenaline in male and females, and to vasopressin and a depolarizing potassium solution in males only. The *in vitro* perfusion preparation used in these studies was separated from other *in vivo* systems known to regulate vascular tone, suggesting therefore, that a change in receptor binding or alterations in the production of local vasorelaxants/constrictors underlies the increased vascular sensitivity. Glucocorticoids may regulate the synthesis of vasoactive compounds, such as prostaglandins or nitric oxide, which in turn modulate peripheral vascular reactivity. Indeed, inhibition of nitric oxide synthesis (Wallerath *et al.* 1999) and decreased levels of both prostaglandin and its mediator adenylate cyclase (Handa *et al.* 1984; Handa *et al.* 1983) have been demonstrated in adult rats with DEX-induced hypertension. Similarly increased mesenteric vasculature sensitivity to noradrenaline has been observed in rats treated with low doses of DEX (Russo *et al.* 1990). Notably, these animals were at a pre-hypertensive stage, and had alterations in both prostaglandin synthesis and in the neuronal uptake of noradrenaline. The gender differences noted in the mesenteric vasculature responses of DEX-treated offspring to both vasopressin and potassium chloride are intriguing, and could possibly reflect changes in vascular reactivity during the reproductive cycle (Dalle Lucca *et al.* 2000), and the effect of sex steroids on vascular smooth muscle sensitivity (Garcia-Villalon *et al.* 1996). Interestingly, as in this study, gender has been previously demonstrated to have no effect on the sensitivity to noradrenaline in both the rat tail artery and aorta (Garcia-Villalon *et al.* 1996; Fulton *et al.* 2002).

Antenatal glucocorticoids have well documented and very substantial immediate benefits in threatened pre-mature labour (Crowley 2003; Crowley 2000). However, there is ever increasing interest regarding the non-pulmonary effects of glucocorticoid exposure to the fetus (O'Regan *et al.* 2001). Indeed, recent data have suggested that a short antenatal glucocorticoid exposure in preterm infants is associated with increased blood pressure at adolescence (Doyle *et al.* 1999). The present study, though somewhat small in sample number and duration, advances our knowledge of the nature and mechanisms of glucocorticoid-induced alterations in postnatal blood pressure. As the prenatal glucocorticoid exposure models extend our understanding of the fetal origins phenomena, it is clear that there is a real need for replication and extended studies in humans. Only then, will we be better equipped to understand both the immediate and long-term consequences of antenatal glucocorticoid treatment.

Discussion

Mechanisms of glucocorticoid programmed disease

7.1 Background and aims

When glucocorticoids were first employed therapeutically almost 50 years ago, they were administered with great gusto, including during pregnancy. From the outset, results showed that the offspring of women treated with glucocorticoids when pregnant had reduced birth weight, an effect that is reflected in numerous experimental animals (reviewed in O' Regan *et al.* 2001). Repeated global epidemiological studies have linked low birth weight and other markers of restrained growth in early life with a considerable increased risk of hypertension, type II diabetes and ischaemic heart disease (Barker 1998). Given the growth-retarding effects of glucocorticoids, their key role in determining the trajectory of organ maturation and their direct hypertensive and hyperglycaemic effects *in vivo*, some investigators questioned whether glucocorticoids might underlie these epidemiological links (Edwards *et al.* 1993; Seckl *et al.* 1999). Subsequent studies in rats, sheep and guinea pigs showed that brief glucocorticoid exposure during specific 'developmental windows' of foetal life could permanently programme or 'hardwire' tissue responses, leading to persistent elevations of blood pressure and blood glucose and insulin levels in adult life. Now, research is focused on identifying the underlying mechanisms that are involved in the glucocorticoid programming of disease.

To this end, research in this thesis was aimed at primarily determining the nature and mechanism(s) of glucocorticoid programmed hypertension, and to further determine whether programmed cardiovascular and metabolic events are sexually dimorphic. It further examined the impact of dietary manipulations, and environmental noise stress, on prenatally-treated offspring phenotypes.

7.2 Gender specific programming of cardiovascular and metabolic abnormalities

7.2.1 Metabolic phenotypes

The results presented in Chapter 4 of this thesis adds further weight to the hypothesis that *in utero* over-exposure to glucocorticoids programmes increased hepatic glucose output, thereby producing glucose intolerance and insulin resistance throughout adult life. Evidence is presented that ***none*** of these effects occur in female offspring, even though treatment involved the same gestational window and indeed dams as the males, and birth weight was similarly reduced in both sexes.

Analogous findings have been made in an alternative model of programmed insulin resistance i.e. only the male offspring of protein restricted dams have elevated PEPCK activity (Desai *et al.* 1997b) and are insulin resistant (Desai *et al.* 1997a; Sugden & Holness 2002). Interestingly, the two prenatal challenges may be related as dietary protein restriction reduces placental 11β-HSD type II, allowing excess foetal glucocorticoid exposure (Langley-Evans *et al.* 1996).

Furthermore, the evidence that only DEX-treated male offspring display a hyperactive HPA-axis provides one mechanism to explain the sex-specific cause of their glucose dyshomeostasis.

The male-specific metabolic response to prenatal DEX programming may be important in relation to other studies showing increased vulnerability of male rats, compared to females, to the later development of obesity (Anguita *et al.* 1993), hypercholesterolaemia (Lucas *et al.* 1996) and triacylglycerolaemia (Lucas *et al.* 1996) as a result of early protein restriction. At each stage during adulthood, the DEX-treated males in our studies were considerably heavier than their female siblings, despite being similarly intra-uterine growth retarded. Thus, could the combination of IUGR and excess weight gain in adult life be particularly detrimental to male rats? This suggestion would

be consistent with observations in humans that show IUGR men to be more insulin resistant than women (Phillips *et al.* 1994). It is further known that the distribution of body fat is important in the etiology of insulin resistance, and men have greater fat deposition around the waist (intra-abdominal obesity) associated with greater insulin resistance (Ferrannini 1995; Ludvik *et al.* 1995). Interestingly, corticosterone treatment of male rats has been demonstrated to uniquely increase intra-abdominal fat depots (Rebuffe-Scrive *et al.* 1992), though the effect of prenatal DEX on body fat distribution remains to be determined in our model. Additionally, it is only women with a relative hyperandrogenicity that are statistically associated with a centralisation of body fat and insulin resistance (Nilsson *et al.* 1998). Thus, it is also possible that the higher male androgen levels drive body fat centralisiation and promote insulin resistance. Consistent with this hypothesis, female rat pups imprinted with androgens display increased intra-abdominal fat depots and insulin resistance (Nilsson *et al.* 1998). Furthermore, it is tempting to speculate that the DEX-treated male increase in insulin resistance may operate in tandem with their higher basal glucocorticoid levels to increase PEPCK activity (Friedman *et al.* 1997; Reshef *et al.* 1970).

7.2.2 Cardiovascular phenotypes

Data presented in Chapter 4 also reinforces the notion that prenatal DEX treatment results in adult offspring with elevated blood pressure (Benediktsson *et al.* 1993; Levitt *et al.* 1996b). In contrast to previous studies (Benediktsson *et al.* 1993), the evidence presented here suggests that this characteristic was female-specific.

Similar female-specific elevations in blood pressure have been made in other prenatal glucocorticoid (Ortiz *et al.* 2003) and dietary (Khan *et al.* 2003) programming models, although as illustrated by Chapter 6 and others (Ortiz *et al.* 2003), these data may reflect the inherent problems of single time-point measurement techniques.

Whatever the basis for the sex difference in this study, prenatal glucocorticoid administration resulted in generalised activation of the RAS in the hypertensive female but not the normotensive male littermate offspring; with the higher hepatic angiotensinogen mRNA levels and consequent elevation in plasma angiotensinogen concentrations probably driving female hypertension.

Angiotensinogen is a key step determining RAS activation in rodents (Tamura *et al.* 1996), has been implicated to underlie in part the glucocorticoid programmed hypertension in sheep (Dodic *et al.* 2001), and is a strong candidiate gene for hypertension in humans (Corvol *et al.* 1999; Martinez *et al.* 2002; Procopciuc *et al.* 2002).

Importantly, circulating oestradiol did not vary physiologically between the groups of prenatally treated offspring, though the naturally higher female levels could account for the gender differences in RAS programming.

7.3 Nature and mechanisms of glucocorticoid programmed hypertension

Results presented in Chapter 6 illustrate that prenatal DEX during the last week of pregnancy actually results in ***lower*** basal blood pressure in the adult offspring; with the previously reported hypertensive phenotype only being noted when these offspring are subjected to any stressor, regardless of its apparent banality. Moreover, DEX-treated offspring maintain this stress-induced hypertension for longer following removal of the stressor. These hypertensive responses are mediated by alterations in the responsivity of the SNS, being ameliorated by the inhibition of catecholamine synthesis, and further exaggerated by the promotion of systemic catecholamine release. Additionally, evidence is presented that DEX-treated offspring display greater sensitivity to a variety of vasoconstrictors in the isolated mesenteric vasculature.

Collectively, all the findings in this thesis reinforce the early life origins of disease hypothesis. They provide further evidence, and more importantly, mechanisms for the programming of metabolic and cardiovascular pathophysiology, over and above those derived from epidemiological associations. Furthermore, genes identified as being differentially expressed between prenatally glucocorticoid treated and control animals (e.g. angiotensinogen), could become targets for therapeutic intervention. By targeting these genes in individuals deemed at risk because of low weight or thinness at birth, it may be possible to ameliorate or prevent the development of adulthood hypertension and insulin resistance. For example, in rodents, early postnatal treatment with an inhibitor of the RAS prevents the subsequent development of adulthood hypertension in the offspring of protein restricted dams (Sherman & Langley-Evans 1998).

These findings may be of relevance to humans, as antenatal glucocorticoids have extensive therapeutic use prenatally. Despite their apparent benefits to the preterm infant (Crowley 2000; Crowley 2003), there is ever increasing interest regarding the non-pulmonary effects of glucocorticoid exposure to the foetus (O' Regan *et al.* 2001). Indeed, recent data have suggested that a short antenatal glucocorticoid exposure in preterm infants is associated with increased blood pressure at adolescence (Doyle *et al.* 1999). The present thesis provides novel information regarding the nature and mechanisms of glucocorticoid-induced alterations in postnatal blood pressure. Moreover, it may shape future clinical studies, by highlighting the absolute necessity for ambulatory BP monitoring, as to avoid stress artifacts or 'white-coat' hypertension in low birth weight subjects. Alternatively, it is tempting to speculate that these findings provide a mechanistic basis for 'white-coat' hypertension; and it would be interesting to elucidate whether those susceptible to the condition are in fact 'programmed'. As the 'disturbance' stress studies in Chapter 6 illustrate, prenatally DEX-treated animals are unable to adapt to their environment, and ambulatory BP measurements would provide valuable and detailed BP profiles about low birth weight human subjects as they go about their everyday lives. As the prenatal glucocorticoid exposure model continues to extend our understanding of the early-life origins phenomena, it is clear that there is a

real need for replication and extended studies in humans. Only then, will we be better equipped to understand both the immediate and long-term consequences of antenatal glucocorticoid treatment. In the interim, the further evidence presented in this thesis recommends that caution is required in the use of these interventions; whilst some glucocorticoid is good, more is not necessarily better.

7.4 Dietary effects on programming

Data presented in Chapter 5 revealed that a 4-fold reduction in dietary sodium results in hypertension in prenatally vehicle treated animals; and supports a role for both the HPA and RAS in mediating this. Conversely, whilst a lower sodium diet intensified the pre-existing RAS dysregulation in DEX-treated offspring, it did not exacerbate their programmed blood pressure phenotype, and it is tempting to speculate that ANP may play a role in preventing further hypertension (see section 5.3; Weidmann *et al.* 1986). Furthermore, evidence is presented that acute exposure to a low sodium diet is sufficient to cause glucose intolerance and insulin resistance in adult rats, irrespective of their prenatal treatment. By activating the RAS, a low sodium diet can reduce blood flow to the liver (Ichikawa *et al.* 1979) thereby decreasing insulin clearance (Meland *et al.* 1994; Prada *et al.* 2000); thus providing one mechanism to explain this phenomenon.

7.5 Perinatal stress effects on programming

Results presented in Chapter 3 clearly illustrate the power of perinatal environmental noise stress to alter the phenotype of prenatally vehicle and DEX- treated offspring (see Table 3-2). The programming effects of stress alone on metabolic and cardiovascular physiology remain to be elucidated, but are most probably mediated through increased glucocorticoid secretion, and would therefore be expected to exacerbate the effects of prenatal vehicle or DEX treatment.

Vehicle and DEX-treated birth phenotypes normally observed in this paradigm (see Table 3-2) were altered, and the previously demonstrated differences in adult phenotype were completely abolished. Given that the birth phenotypes were affected, it is likely that the environmental noise stress was present during the prenatal period. Reviewing the absolute values for each investigated physiological parameter indicates that the phenotype of vehicle-treated offspring had been shifted towards that of the DEX-treated animals, with little if any deviation of the latter (see Table 3-2). This not only supports the above assertion, but further implies that there is an 'upper-limit' for the programming effects of stress and glucocorticoids.

7.6 Signposting future frontiers

Excitingly, many frontiers remain to be investigated before we fully comprehend the mechanisms of prenatal glucocorticoid programmed disease; and elements of the work presented in this thesis not only signpost these areas, but also indicate those worth revisiting.

Amongst the latter, include aspects of the work presented in Chapter 5. Repetition of many of the measurements under the initially proposed dietary conditions, with the addition of: a high (3%) sodium diet, plasma AngII and ANP measurements, and radiotelemetric blood pressure monitoring, would be instructive in further elucidating the phenotype of the DEX-programmed rat.

Data presented in this thesis strengthen previous results that the last week of gestation is the crucial 'developmental window' in the rat during which glucose dyshomeostasis (Nyirenda *et al.* 1998) and hypertension (Levitt *et al.* 1996a) can be programmed. However, there is scope for this window to be further defined, as administration of DEX during the last two days of pregnancy alone did not result in adult hyperglycaemia (Nyirenda *et al.* 2001), whereas constant treatment throughout pregnancy has resulted in hypertension (Benediktsson *et al.* 1993). Epidemiological studies have suggested that

infant size and proportion, and the nature of subsequent programmed events varies according to the trimester during which the insult occurred (Osmond & Barker 2000; Roseboom *et al.* 2001). The nature of the systems susceptible to DEX treatment during discrete developmental windows will be dependent on the foetal gene expression and biochemistry at that time, and could possibly underlie some of the observed sex differences.

Further elucidation of the role of the SNS in this model is also warranted. Charaterisation of α- and β-adrenoreceptors in prenatally treated offspring would establish whether receptor subtype(s) underlie the increased catecholaminergic responsitivity associated with prenatal DEX-treatment. Furthermore, given the key role of the SNS in regulating the internal milieu, it would be of great interest to ascertain whether SNS dysfunction underlies other programmed phenotypes. For example, does increased programmed SNS responsivity, associated with prenatally DEX-treated offspring, contribute to their glucose dyshomeostasis? Treatment with the catecholamine synthesis inhibitor, reserpine, prior to commencement of an OGTT could address this.

Given the importance of programmed alterations in both the peripheral RAS and SNS to the cardiovascular phenotype of prenatally DEX-treated offspring, there is a clear need to extend investigations to those central regions governing blood pressure control. In the brain, there is great interplay between both systems, with AngII being a well documented stimulator of the SNS (Segar *et al.* 2001). On this basis, an obvious first candidate to characterise would be AngII receptors in central sympathetic areas.

Several epidemiological studies advocate a positive correlation between maternal BP during pregnancy and BP in children (Himmelmann *et al.* 1994; Himmelmann 1994). Examination of this association in the DEX-model would obviously be worthwhile, in order to determine if prenatal treatment has any adverse affects on maternal BP, and if it is these alterations that contribute either wholly or in part to offspring BP, rather than the prenatal treatment per se. Using radiotelemetry, it would be possible to accurately profile

maternal BP prior to pregnancy, during prenatal treatment and the early postnatal period; subsequent radiotelemetric BP recordings could then be made in her adult offspring.

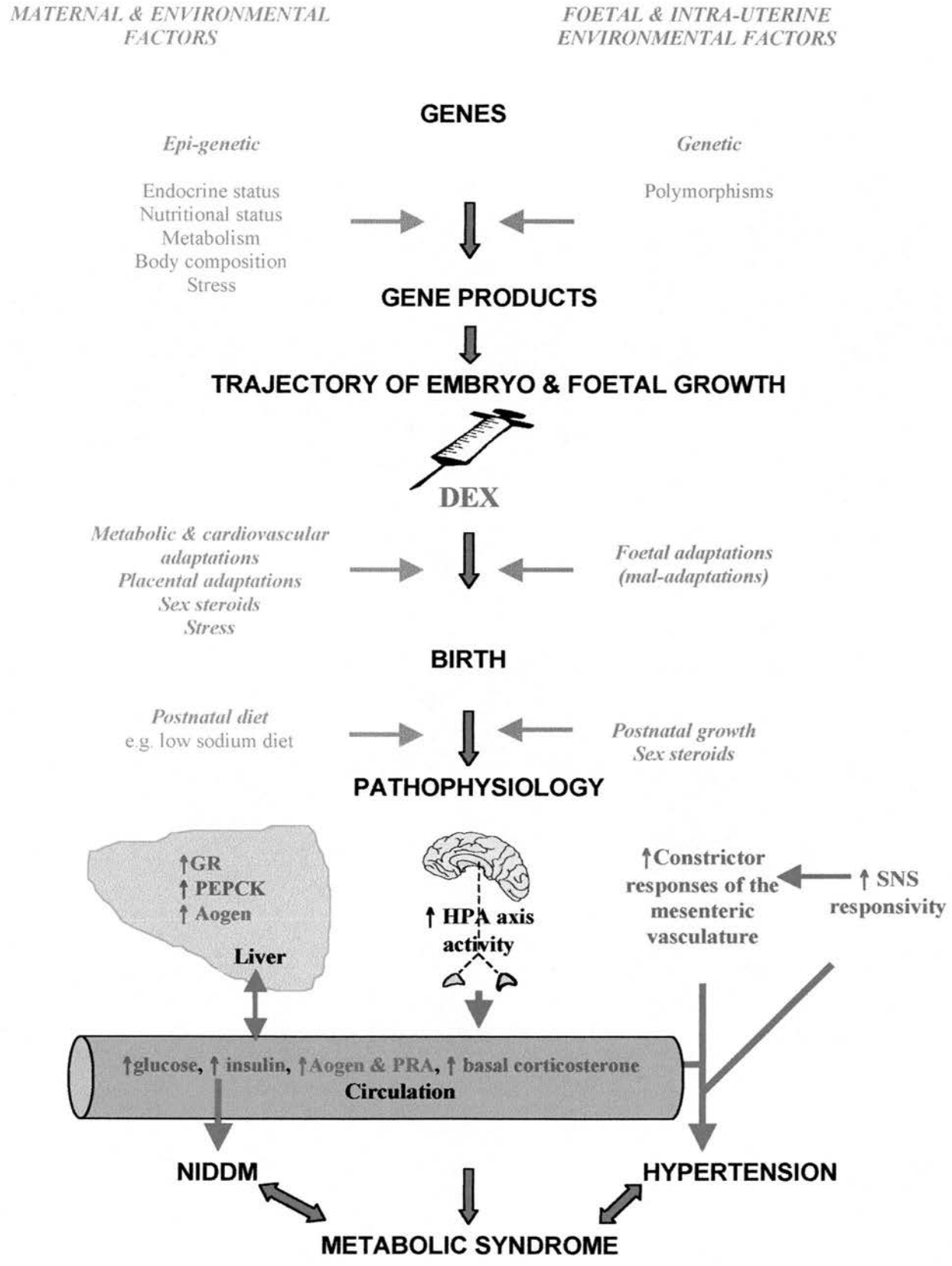
The emerging role of gender in determining programmed outcomes is very intriguing, and as previously discussed (see section 7.2), the mechanisms underlying this sexual dimorphism in the effect of prenatal DEX on postnatal physiology clearly requires further investigation.

Additionally, there remain several key topics to be addressed in our prenatal glucocorticoid over-exposure model. As yet, no-one has examined whether RAS inhibitors administered early in the postnatal period could ameliorate or prevent later offspring hypertension, as in other prenatal programming models (Sherman & Langley-Evans 1998). Furthermore, it has yet to be determined whether programmed alterations in the pancreas of prenatal DEX-treated rats contribute to their glucose dyshomeostasis. Epidemiological studies and other programming studies in rodents have associated low birth weight with impaired immune function (McDade *et al.* 2001a; McDade *et al.* 2001b; Bakker *et al.* 1998), and renal impairment (Eriksson *et al.* 2000; Ortiz *et al.* 2003). Whether these abnormalities are associated with our prenatal DEX programming model remains to be investigated.

Finally, recent evidence further emphasising the importance of early life events suggest that they may also affect the health of subsequent generations. A clear inverse correlation has been demonstrated between maternal birth weight and offspring BP that is independent of offspring birth weight (Barker *et al.* 2000). Moreover, in rodents, intrauterine maternal malnutrition has resulted in F_2 adult offspring with pronounced insulin resistance (Martin *et al.* 2000). The potential for prenatal DEX to influence the metabolic and cardiovascular physiology of subsequent generations is the focus of current research in our laboratory.

7.7 Summary

Much of the world's population is challenged by increasing rates of adult-onset diabetes and cardiovascular disease. Prevention of these chronic illnesses requires a search for the origins of disease extending beyond the traditional limits of medical research and practice. This thesis adds weight to the hypothesis that *in utero* over-exposure to glucocorticoids can programme alterations in metabolic and cardiovascular physiology that persist throughout adult life (summarised in **Figure 7-1**). Moreover, it seems that prenatal DEX treatment produces hypertension that is specifically stress-induced, and support a role for both the RAS and SNS in mediating this. Additionally, it appears that the programming of cardiovascular physiology may reflect distinct processes in each gender, whilst the programming of metabolic physiology is male specific. These findings may prove valuable in directing future scientific and clinical investigations that ultimately establish beneficial therapeutic interventions.


MATERNAL & ENVIRONMENTAL FACTORS
FOETAL & INTRA-UTERINE ENVIRONMENTAL FACTORS
GENES
Epi-genetic
Genetic
Endocrine status
Nutritional status
Metabolism
Body composition
Stress
Polymorphisms
GENE PRODUCTS
TRAJECTORY OF EMBRYO & FOETAL GROWTH
DEX
Metabolic & cardiovascular adaptations
Placental adaptations
Sex steroids
Stress
Foetal adaptations (mal-adaptations)
BIRTH
Postnatal diet
e.g. low sodium diet
Postnatal growth
Sex steroids
PATHOPHYSIOLOGY
↑GR
↑ PEPCK
↑ Aogen
Liver
↑ HPA axis activity
↑Constrictor responses of the mesenteric vasculature
↑ SNS responsivity
↑glucose, ↑ insulin, ↑Aogen & PRA, ↑ basal corticosterone
Circulation
NIDDM
HYPERTENSION
METABOLIC SYNDROME

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